Molecular biological and biochemical investigations of prenyltransferases from different *Aspergillus* species

Molekularbiologische und biochemische Untersuchungen zu Prenyltransferasen aus verschiedenen *Aspergillus* Arten

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Publications and presentations

Publications

1. Pockrandt D, Ludwig L, Fan A, König G M, Li S-M (2012) New insights into the biosynthesis of prenylated xanthones: XptB from *Aspergillus nidulans* catalyses an O-prenylation of xanthones. Chembiochem 13, 2764–2771

2. Pockrandt D, Li S-M (2013) Geranylation of cyclic dipeptides by the dimethylallyl transferase AnaPT resulting in a shift of prenylation position on the indole ring. Chembiochem 14, 2023–2028

3. Pockrandt D, Sack C, Kosiol T, Li S-M (2014) A promiscuous prenyltransferase from *Aspergillus oryzae* catalyses C-prenylations of hydroxynaphthalenes in the presence of different prenyl donors. Appl Microbiol Biotechnol. 2014 DOI: 10.1007/s00253-014-5509-x

Presentations

1. Pockrandt D, Ludwig L, Fan A, König G M, Li S-M. XptB from *Aspergillus nidulans* is an O-prenyltransferase for xanthones. Poster presentation, 1st European Conference on Natural Products, September 2013, Frankfurt, Germany

Share of author contributions

Publication	Authors	Estimated own contribution (%)
New insights into the biosynthesis of prenylated xanthones: XptB from *Aspergillus nidulans* catalyses an O-prenylation of xanthones (published)	Pockrandt D, Ludwig L, Fan A, König G M, Li S-M	65
Geranylation of cyclic dipeptides by the dimethylallyl transferase AnaPT resulting in a shift of prenylation position on the indole ring (published)	Pockrandt D, Li S-M	75
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Candidate

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Supervisor

Abbreviations

4-DMATS	4-dimethylallyltryptophan synthase
A_{600nm}	absorption at 600 nm
A. flavus	*Aspergillus flavus*
A. fumigatus	*Aspergillus fumigatus*
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
A. oryzae	*Aspergillus oryzae*
A. terreus	*Aspergillus terreus*
approx.	approximately
AspGD	Aspergillus Genome Database
BLAST	basic local alignment search tool
bp	base pair
Da	Dalton
dd	double doublet
DMA	dimethylallyl
DMAPP	dimethylallyl diphosphate
DMATS	dimethylallyltryptophan synthase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
e. g.	*exempli gratia*
EI	electron impact ionization
ESI	electrospray ionization
FPP	farnesyl diphosphate
gDNA	genomic DNA
GPP	geranyl diphosphate
His_6	hexahistidine
HPLC	high performance liquid chromatography
HR	high resolution
Hz	hertz

i. e.	*id est*
IPTG	isopropyl-β-D-thiogalactopyranoside
k_{cat}	turnover number
kDa	kilodalton
K_M	Michaelis-Menten constant
LB	lysogeny broth
m	multiplet
Mb	mega base pairs
ml	millilitre
MS	mass spectrometry
N. fischeri	*Neosartorya fischeri*
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
NRPS	non-ribosomal peptide synthetase
PCR	polymerase chain reaction
P. aethiopicum	*Penicillium aethiopicum*
PKS	polyketide synthase
PPi	pyrophosphate
ppm	parts per million
RP	reversed phase
rpm	revolutions per minute
s	singlet
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gelelectrophoresis
t	triplet
v/v	volume per volume
w/v	weight per volume
WHO	world health organisation
x g	times of gravitational acceleration

Summary

Prenylated aromatic compounds are widely distributed in nature and many of these compounds display interesting biological and pharmaceutical activities. The transfer of prenyl moieties to aromatic substrates is catalysed by aromatic prenyltransferases, and the multitude of possibilities for attachment of these moieties gives rise to a large structural diversity. Investigation of the functions of aromatic prenyltransferases would therefore help to elucidate the biosynthetic pathways of those natural compounds in the producing organism, as well as the applicability of these enzymes for chemoenzymatic synthesis of biologically active compounds for pharmaceutical purposes.

Four putative prenyltransferase genes of the dimethylallyltryptophan synthase (DMATS) superfamily from *Aspergillus nidulans* (*A. nidulans*) were investigated as one part of this thesis. The genes *xptA*, *xptB*, ANID_10289 and ANID_11080 were cloned, and the two putative xanthone prenyltransferase genes *xptA* and *xptB* were overexpressed in *Escherichia coli* (*E. coli*). Both recombinant prenyltransferases were purified to near homogeneity using affinity chromatography with Ni-NTA and investigated biochemically. In this thesis, the enzyme XptB was confirmed to function as a xanthone prenyltransferase, catalysing a regiospecific O-prenylation of several xanthones. The enzyme products were isolated on high performance liquid chromatography (HPLC) and analysed by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). Another previously postulated substrate, the benzophenone arugosin H, was not accepted by XptB under the tested conditions. No enzyme activity was detected for the second enzyme XptA with postulated or potential substrates.

Reports of the enzyme VrtC from *Penicillium aethiopicum* (*P. aethiopicum*) implicated that some members of the DMATS superfamily might not be restricted to the usual prenyl donor dimethylallyl diphosphate (DMAPP, C_5-unit). VrtC was shown as the first known member of the DMATS prenyltransferases, which accepts geranyl diphosphate (GPP, C_{10}-unit), but not DMAPP, for prenylation of a naphthacenedione substrate. Therefore, the acceptance of prenyl donors, such as GPP and farnesyl diphosphate (FPP, C_{15}-unit), was investigated in this thesis with the prenyltransferases 7-DMATS and CdpNPT, both from *Aspergillus fumigatus* (*A. fumigatus*), as well as CdpC3PT and AnaPT, both from *Neosartorya fischeri* (*N. fischeri*). It was found that AnaPT accepted, in addition to DMAPP, also GPP as prenyl donor. Furthermore, a shift of the prenylation position was observed. Instead of a reverse C3α-prenylation with DMAPP, C6- and C7-geranylations of tryptophan-containing cyclic dipeptides with GPP were

demonstrated in this thesis. This was the first report of the acceptance of both DMAPP and GPP by a member of the DMATS superfamily. The observed shift of the prenylation position by changing the prenyl donor expands the applicability of AnaPT for the production of prenylated compounds.

As last part of this thesis, the putative prenyltransferase genes AO090102000322, AO090701000600 and AO090020000527 from *Aspergillus oryzae* (*A. oryzae*) were cloned. The recombinant protein BAE61387, encoded by AO090102000322, was successfully overproduced and purified from *E. coli*. Analysis of neighbouring genes in the genome of *A. oryzae* revealed no backbone genes for the biosynthesis of a possible natural substrate of BAE61387. The enzyme BAE61387 was therefore incubated with a large number of compounds that are known substrates of enzymes from the DMATS superfamily. Activity was clearly observed in incubation mixtures with hydroxynaphthalenes. Interestingly, these unnatural substrates, which are accepted by several members of this enzyme class, were accepted by BAE61387 in the presence of all three prenyl donors DMAPP, GPP and FPP. The acceptance of all these prenyl donors for regiospecific C-prenylations of hydroxynaphthalenes has never been shown for a member of this enzyme superfamily prior to this work.

Zusammenfassung

Prenylierte aromatische Verbindungen sind in der Natur weit verbreitet und viele dieser Stoffe besitzten interessante biologische und pharmazeutische Aktivitäten. Die Übertragung der Prenylreste auf aromatische Substrate wird durch aromatische Prenyltransferasen katalysiert und die Vielzahl der Verknüpfungsmöglichkeiten der Prenylreste ermöglicht eine hohe strukturelle Diversität prenylierter Naturstoffe. Untersuchungen zur Funktion von aromatischen Prenyltransferasen geben einen Einblick in die Biosynthesewege dieser Naturstoffe und bieten diese Enzyme als Werkzeuge in der chemoenzymatischen Herstellung von biologisch aktiven Substanzen für pharmazeutische Anwendungen an.

Vier putative Prenyltransferasegene der Dimethylallyltryptophan Synthase (DMATS) Superfamilie aus *Aspergillus nidulans* (*A. nidulans*) wurden als ein Teil der vorliegenden Arbeit untersucht. Die Gene *xptA*, *xptB*, ANID_10289 und ANID_11080 wurden kloniert, und die beiden putativen Xanthon-Prenyltransferasegene *xptA* und *xptB* wurden in *Escherichia coli* (*E. coli*) überexprimiert. Die rekombinanten Enzyme wurden mittels Affinitätschromatographie mit Ni-NTA aufgereinigt und biochemisch im Hinblick auf ihre Enzymaktivität und Substratspezifität untersucht. In der vorliegenden Arbeit konnte das Enzym XptB als Xanthon-Prenyltransferase bestätigt werden, welche die regiospezifische O-Prenylierung von einigen Xanthonen katalysiert. Die enzymatischen Produkte wurden mittels *high performance liquid chromatography* (HPLC) isoliert und mithilfe von Kernresonanzspektroskopie (NMR) und Massenspektrometrie (MS) untersucht. Ein weiteres in der Literatur postuliertes Substrat von XptB, das Benzophenon Arugosin H, wurde unter den getesteten Bedingungen nicht umgesetzt. Für das zweite aufgereinigte Enzym XptA konnte allerdings keine enzymatische Aktivität, weder in Inkubationsansätzen mit dem postulierten Substrat Variecoxanthon A, noch mit anderen potenziellen Substraten nachgewiesen werden.

Mit dem Enzym VrtC aus *Penicillium aethiopicum* (*P. aethiopicum*) wurde erstmals die Umsetzung einer Naphthacendion-Verbindung mit Geranyldiphosphat (GPP, C_{10}-Einheit) durch eine DMATS-Prenyltransferase beschrieben. Diese Entdeckung führte zu der Annahme, dass nicht alle DMATS-Prenyltransferasen auf den sonst üblichen Prenyldonor Dimethylallyldiphosphat (DMAPP, C_5-Einheit) beschränkt sein müssen. In der vorliegenden Arbeit wurden dazu die bekannten Prenyltransferasen 7-DMATS und CdpNPT, beide aus *Aspergillus fumigatus* (*A. fumigatus*), sowie CdpC3PT und AnaPT, beide aus *Neosartorya fischeri* (*N. fischeri*), auf die Akzeptanz von GPP und Farnesyldiphosphat (FPP, C_{15}-Einheit) untersucht. Von den untersuchten Enzymen konnte für AnaPT, zusätzlich zu DMAPP, eine

Umsetzung von Tryptophan-enthaltenden zyklischen Dipeptiden mit GPP nachgewiesen werden. Für sechs zyklische Dipeptide, für welche mit DMAPP zuvor eine reverse C3α-Prenylierung beobachtet wurde, konnte durch die Verwendung von GPP in der vorliegenden Arbeit die Geranylierung durch AnaPT an den Positionen C-6 und C-7 des Indols nachgewiesen werden. Eine Akzeptanz von sowohl DMAPP als auch GPP durch eine DMATS-Prenyltransferase wurde somit erstmals demonstriert. Die Akzeptanz von DMAPP und GPP, sowie die beobachtete Verschiebung der Prenylierungsposition durch den Einsatz von Prenyldonoren unterschiedlicher Länge, erhöhen die Nützlichkeit dieses Enzyms zur chemoenzymatischen Synthese prenylierter Verbindungen.

Als letzter Teil dieser Arbeit wurden die drei putativen Prenyltransferasegene AO090102000322, AO090701000600 und AO090020000527 aus *Aspergillus oryzae* (*A. oryzae*) kloniert. Das putative Produkt BAE61387 von AO090102000322 wurde in *E. coli* überproduziert und das rekombinante Enzym erfolgreich aufgereinigt. Durch Sequenzanalysen konnten im Genom von *A. oryzae* in der Nachbarschaft zu AO090102000322 keine Gene identifiziert werden, welche Aufschluss über ein mögliches natürliches Substrat von BAE61387 geben könnten. Das erhaltene Protein wurde daher mit zahlreichen Substraten von bekannten Enzymen der DMATS-Superfamilie inkubiert. Eine enzymatische Aktivität konnte in Inkubationsansätzen mit einigen Hydroxynaphthalin-Derivaten beobachtet werden. Allerdings wurden diese unnatürlichen Substrate, welche von einigen Mitgliedern der DMATS-Prenyltransferasen akzeptiert werden, in Gegenwart von DMAPP, GPP und FPP umgesetzt. Die Akzeptanz der drei Prenyldonoren DMAPP, GPP und FPP zur regiospezifischen C-Prenylierung von Hydroxynaphthalin-Derivaten durch eine DMATS-Prenyltransferase wurde zuvor noch nicht demonstriert.

1 Introduction

1.1 Ascomycota

The order ascomycota, or sac fungi, represents one of the large orders of the fungal kingdom (Feofilova 2001). The ascomycetes are characterised by the shape of their reproductive structures, the tube-shaped asci, bearing haploid ascospores. The growth habit of ascomycetes is mostly that of a mycelium formed by massed branched hyphae, although the order also contains single-cell yeasts. Ascomycetes undergo different alternation of generations, in a sexual state (teleomorph), a diploid organism produces the meiotic ascospores, but reproduction can also be facilitated by the production of mitotic conidia by the asexual state of the fungus (anamorph). The sexual form of reproduction is unknown from several ascomycetes, those fungi have been referred to as the artificial taxon "fungi imperfecti" (Adrio & Demain 2003). The ascomycetes include several mould fungi of the family Trichocomaceae, containing the genera *Aspergillus*, *Neosartorya* and *Penicillium*. Mould fungi from these genera are of large importance for mankind, for example, in their role as pests or pathogens in food production and healthcare. However, apart from their deleterious effects, many moulds exhibit unique properties, which make these organisms interesting for research and applications in food industry and life sciences (Kuck & Bohm 2013).

1.2 Secondary metabolism of mould fungi

One characteristic feature of mould fungi is the occurrence of an extensive secondary metabolism. Secondary metabolites are in general compounds that are produced by an organism without elemental necessity for growth, survival or reproduction of the producing organism (Vining 1990). However, it is widely accepted that the production of secondary metabolites gives these organisms an ecological advantage for their survival in a competitive environment (Losada *et al.,* 2009). Secondary metabolites derive from primary metabolite scaffolds and their production can draw vast amounts of chemical energy. In the evolution, mould fungi developed an extensive secondary metabolism and the ability to produce some potent mycotoxins for protection against fungivory or mycophagy (Rohlfs *et al.,* 2007). The production of these compounds often correlates with a specific developmental stage. For example, the production of many mycotoxins or pigments correlates with sporulation (Calvo *et al.,* 2002). Numerous secondary metabolites and mycotoxins exert some specific biological and pharmaceutical activities and are therefore of interest for pharmaceutical research and applications (Demain 1999; Demain & Sanchez 2009). In the biosynthesis of many fungal secondary metabolites,

non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), NRPS-PKS-hybrid enzymes or terpene cyclases synthesize the structural backbones (Inglis *et al.,* 2013; Khaldi *et al.,* 2010). They synthesize the five large compound classes, *i*. *e*. non-ribosomal peptides, indole alkaloids, polyketides, peptide-polyketide-hybrid compounds and terpenes, respectively (Hoffmeister & Keller 2007; Khaldi *et al.,* 2010). The structural backbones can be further modified by many enzymes, giving rise to the large structural diversity of secondary metabolites from ascomycetes. An important enzyme class from ascomycetes that introduce such modifications are prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily. The DMATS prenyltransferases catalyse the transfer of prenyl moieties from prenyl diphosphates, *e*. *g*. dimethylallyl diphosphate (DMAPP), to aromatic compounds, mainly indole derivatives. This thesis deals with enzymes of the DMATS superfamily from different *Aspergillus* species and the resulting prenylated aromatic compounds.

On the genetic level, mould fungi show a further characteristic feature, the clustering and coregulation of genes involved in the biosynthesis of secondary metabolites (Bok *et al.,* 2006; Palmer & Keller 2010; Sanchez *et al.,* 2012). With the use of bioinformatic approaches, it is therefore often possible to infer a gene cluster responsible for the production of a given compound by identifying the genes necessary for the biosynthesis of the respective structural backbone and modifying enzymes. It is known for several moulds from the genus *Aspergillus* with available genomic sequences, that they possess a genetic potential for the production of many yet unknown compounds. These cryptic compounds may have interesting biological and pharmaceutical activities (Bok *et al.,* 2009; Hertweck 2009; Yin & Keller 2011). The elucidation of these pathways and compounds is a focus of actual scientific approaches from different disciplines of natural science.

1.3 The genus *Aspergillus*

The genus *Aspergillus* contains many moulds with large importance for mankind. The genus contains about 350 species, which are characterised by the unique aspergillum-shape of their conidiophores (Bennett 2009). These fungi are widely distributed around the world and mostly exhibit a saprobiontic nutrition from decaying or dead organic matter, and therefore play an important role as decomposers in ecological pathways. The genus encompasses several mould fungi that are capable of the production of potent mycotoxins. These mycotoxins can exert harmful effects on animals and humans and are often synthesized in correlation with sporulation of a given mould fungus (Keller *et al.,* 2005). *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus*, for example, produce the cancerogenic aflatoxins (**1**) and sterigmatocystin (**2**) that

are widely occurring contaminants of agricultural products and for which avoidance strict safety measures are employed (Figure 1.1) (Frisvad *et al.,* 2006; Hesseltine *et al.,* 1966; Kabak *et al.,* 2006). Another example of food contaminants produced by *Aspergillus* species is the ochratoxins (**3**). Ochratoxins also exert cancerogenic and nephrotoxic effects in animal experimentation (el & Atoui 2010). *Aspergillus fumigatus* (*A. fumigatus*) produces fumagillin (**4**) with neurotoxic properties (Lin *et al.,* 2013). Gliotoxin (**5**) is another example, which shows cytotoxic effects, and is produced by *Aspergillus terreus* (*A. terreus*) and *A. fumigatus* (Stanzani *et al.,* 2005). The world health organisation (WHO) estimated that in 1999 a quarter of the world's total food production was lost due to contamination with mycotoxins, underlining the importance of these organisms as pests for world nutrition. Some members of the genus *Aspergillus* can also affect human health directly, like *A. fumigatus* or *Neosartorya fischeri* (*N. fischeri*). Inhalation of spores from *A. fumigatus* can cause letal pulmonary aspergillosis in immune-deficient humans (Latge 1999). *N. fischeri*, although rarely occurring as a pathogen, can cause fungal keratitis (Coriglione *et al.,* 1990). In addition, several members of the genus *Aspergillus* produce compounds that can cause allergic effects (Bhetariya *et al.,* 2011).

Apart from their deleterious effects, many of these fungi have properties, which make them useful for applications in food industry or biological and pharmaceutical research. Many of the compounds or enzymes produced by *Aspergillus* species are of enormous use for mankind. *Aspergillus niger* (*A. niger*) and *Aspergillus tubingensis* for example, are important sources of citric acid, lipases and amylases for the food industry (Dhillon *et al.,* 2011; Frisvad *et al.,* 2011; Horn *et al.,* 2013). *Aspergillus oryzae* (*A. oryzae*) and *Aspergillus sojae* are used safely for hundreds of years in asian traditional and industrial fermentation processes (Kobayashi *et al.,* 2007). Examples of secondary metabolites from mould fungi with important biological and pharmaceutical activities are the penicillins, *e. g.* penicillin G (**6**) produced by *Penicillium chrysogenum* and *Aspergillus nidulans* (*A. nidulans*) (MacCabe *et al.,* 1990). Cyclosporin (**7**), an immune-suppressing agent, and the cholesterol-lowering lovastatin (**8**) are further important compounds produced by *A. terreus* (Manzoni & Rollini 2002; Sallam *et al.,* 2003). *A. fumigatus* produces indole alkaloids, like the tryprostatins A (**9**) and B (**10**), with cytotoxic effects against several human cancer cell lines (Cui *et al.,* 1995; Kondoh *et al.,* 1998; Zhao *et al.,* 2002). *A. nidulans* serves as an important model organism in the fields of cell biology, microbiology and genetics. In this thesis, works were carried out with *A. nidulans*, *A. oryzae* and *N. fischeri*, which should be described in more detail.

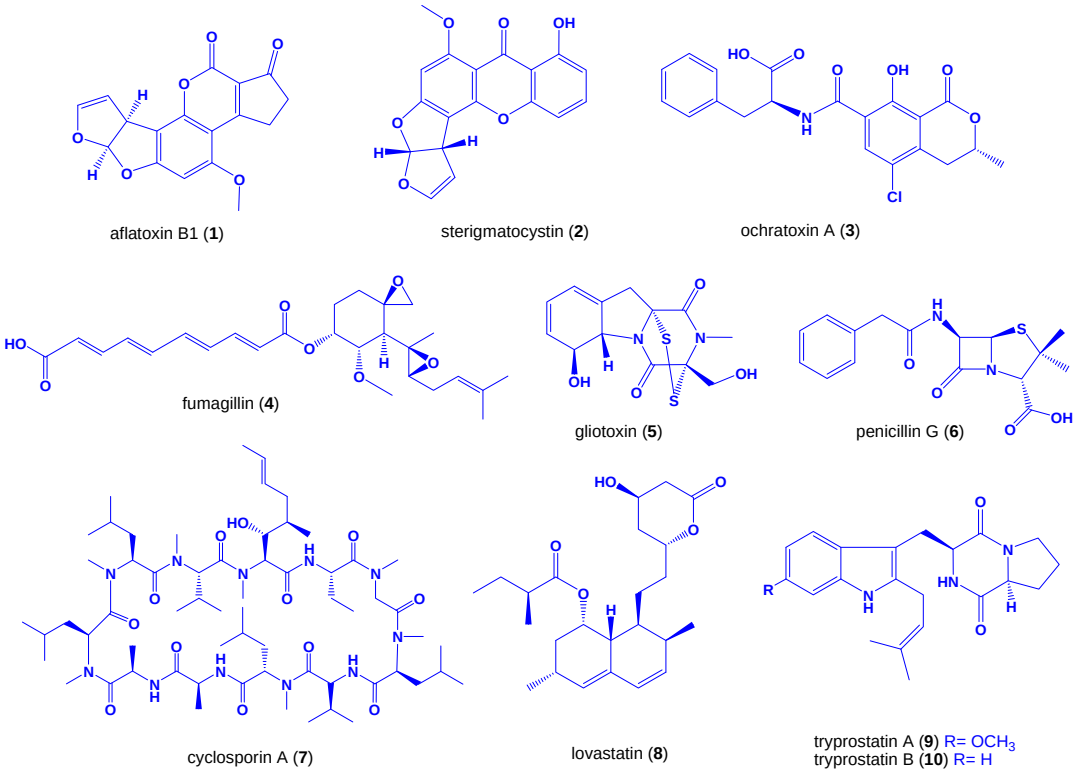


Figure 1.1 Examples of important secondary metabolites produced by different *Aspergillus* species.

1.3.1 *Aspergillus nidulans*

A. nidulans is used as a model organism in the fields of cell biology, genetics and microbiology for over 60 years (Sanchez *et al.*, 2012). Numerous cellular mechanisms, like mitosis (Ukil *et al.*, 2009), regulation of the cell cycle (Osmani & Mirabito 2004), cytoskeleton rearrangements (Prigozhina *et al.*, 2004) and DNA repair mechanisms (Goldman & Kafer 2004) have been investigated with this fungus. *A. nidulans* is non-pathogenic and closely related to industrially and medicinally relevant moulds, like *A. oryzae*, *A. flavus*, *A. niger* and *A. fumigatus*, which makes *A. nidulans* a useful and convenient subject for study. In addition, *A. nidulans* undergoes sexual reproduction under laboratory conditions, which is rarely observed for other *Aspergillus* species. The genome of *A. nidulans* (strain FGSCA4) was sequenced and published in 2003 as a cooperation between the Monsanto Company and the Broad Institute. The genome consist of 30 Mb, with eight chromosomes and contains about 11000 protein-encoding genes (Sanchez *et al.*, 2012). One research focus on *A. nidulans* is the secondary metabolism. After analysis of the genomic sequence, about 50 genes necessary for the biosynthesis of secondary metabolite backbone structures were identified (Khaldi *et al.*, 2010; Szewczyk *et al.*, 2008). These include 24 putative PKS, 12 NRPS and 1 NRPS-PKS-hybrid genes. The genome also encodes seven putative DMATS genes of which four genes were analysed in this thesis (Khaldi *et al.*, 2010; Sanchez *et al.*, 2011; Sanchez *et al.*, 2012). The number of actually isolated and characterised

compounds from *A. nidulans* is relatively low with respect to the genetic potential for the production of secondary metabolites, showing that many secondary metabolism genes are not expressed under laboratory conditions (Bergmann *et al.*, 2007; Szewczyk *et al.*, 2008). Known compounds isolated from *A. nidulans* and the teleomorph *Emericella nidulans* are, for example, the prenylated xanthones variecoxanthone A (**11**), emericellin (**12**), shamixanthone (**13**) and epishamixanthone (**14**) (Sanchez *et al.*, 2011), as well as the prenylated benzophenones arugosin H (**15**), A (**16**) and B (**17**) (Figure 1.2) (Kralj *et al.*, 2006). This thesis deals with the biosynthesis of the diprenylated xanthones and benzophenones from *A. nidulans*, which will be described later. Further examples of compounds isolated from *A. nidulans* are the asterriquinones, like terrequinone A (**18**) (Balibar *et al.*, 2007). Asterriquinones show numerous pharmaceutical activities, like antitumor and antiretroviral activities (Schneider *et al.*, 2008). Thus far, with respect for the genetic potential, only a limited number of natural compounds have been isolated and characterised from *A. nidulans*, and its genome still displays a largely untapped resource of genetic information for the assembly of pharmaceutical active compounds (Bok *et al.*, 2006; Szewczyk *et al.*, 2008; von Döhren 2009).

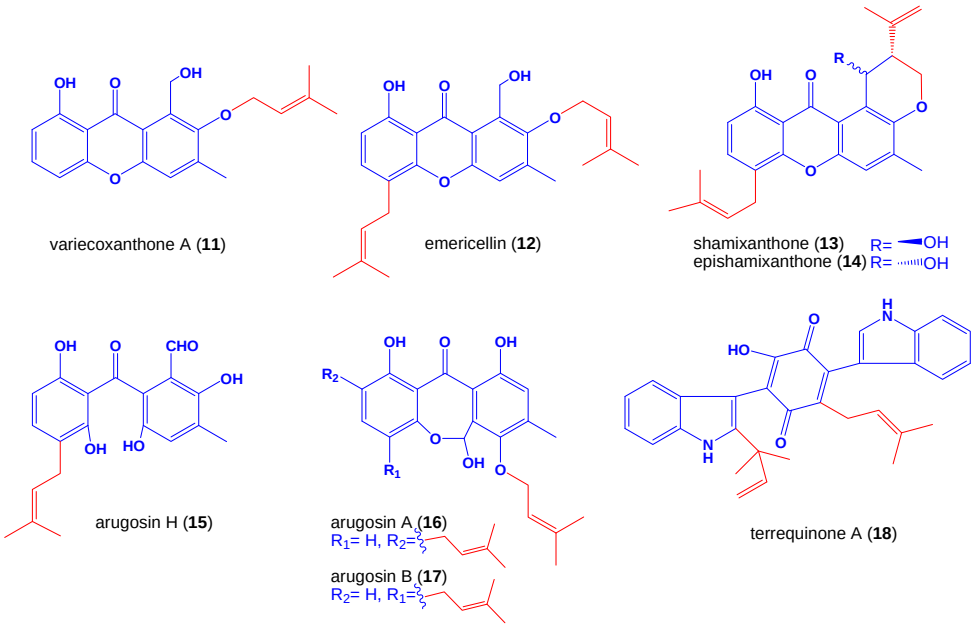


Figure 1.2 Examples of secondary metabolites produced by *Aspergillus nidulans*. Backbone structures are shown in blue and prenyl moieties in red.

1.3.2 *Aspergillus oryzae*

A. oryzae has been used safely for several hundred years in Asia for the fermentation of traditional food products. *A. oryzae* produces and releases a huge amount of enzymes, like amylases and proteases, which are important for these fermentation processes (Kobayashi *et*

al., 2007). The ability to produce and release large amounts of proteins makes *A. oryzae* an ideal host organism for biotechnological purposes, like the production of recombinant eucaryotic proteins (Yoon *et al.*, 2011; Yoon *et al.*, 2013). Furthermore, because of the powerful gene expression machinery, *A. oryzae* is used for the heterologous expression of gene clusters from other ascomycetes, that are only expressed in low quantities in their original hosts (Sakai *et al.*, 2012). The genome of *A. oryzae* (strain RIB40) was sequenced and published in 2005 by members of the *Aspergillus oryzae* Genome Analysis Consortium (Machida *et al.*, 2005). The genome consists of 37 Mb, with eight chromosomes, encoding about 14000 proteins (Galagan *et al.*, 2005). Comparison with the genomes of *A. nidulans* and *A. fumigatus* shows that *A. oryzae* contains about seven Mb additional genetic information (Galagan *et al.*, 2005). Analysis of the additional genes shows that these genes have putative functions in both primary and secondary metabolism (Machida *et al.*, 2005). Investigations of the gene functions indicate that 61 genes are involved in the biosynthesis of secondary metabolite backbone structures (Sanchez *et al.*, 2012). These include 27 PKS, 16 NRPS and 2 NRPS-PKS-hybrid genes. The genome encodes eight putative DMATS genes of which three were analysed in this thesis (Khaldi *et al.*, 2010; Sanchez *et al.*, 2012). An example for a known secondary metabolite from *A. oryzae* is kojic acid (**19**), used as a skin-bleaching agent in cosmetics, which also shows antimicrobial activity (Figure 1.3) (Sanchez *et al.*, 2012). Another example is α-cyclopiazonic acid (**20**), which exhibits inhibitory activity against Ca^{2+}-ATPase, but it is not produced by the genome reference strain RIB40 (Sanchez *et al.*, 2012). In addition, several other analysed *A. oryzae* strains contain the genetic information necessary for the production of mycotoxins, like aflatoxin (**1**) and the tremorgenic indole alkaloid aflatrem (**21**). However, no culture conditions have been established for their production (Akao *et al.*, 2007; Nicholson *et al.*, 2009). Despite the homology of 99.5 % on the genetic level between *A. oryzae* and the notorious pathogenic *A. flavus*, *A. oryzae* seems to be completely non-pathogenic (Nicholson *et al.*, 2009). Because of the close relationship to *A. flavus* and the absent mycotoxin production, *A. oryzae* is often referred to as the "domesticated" form of *A. flavus* (Geiser *et al.*, 1998).

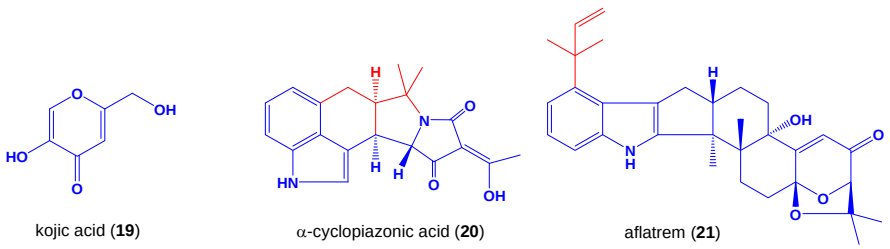


Figure 1.3 Examples of secondary metabolites from different *Aspergillus oryzae* strains. Backbone structures are shown in blue and prenyl moieties in red.

1.3.3 *Neosartorya fischeri*

N. fischeri is a close relative to *A. fumigatus*, which is also human pathogenic (Fedorova *et al.*, 2008). Like *A. fumigatus*, *N. fischeri* can cause a letal pulmonary aspergillosis in immune-deficient humans (Lonial *et al.*, 1997). In addition, *N. fischeri* can infect the skin and cause fungal keratitis, but it is a rather rarely occurring pathogen in comparison to *A. fumigatus* (Coriglione *et al.*, 1990). *N. fischeri* can be found ubiquitary in soil samples and its spores are abundant on agricultural products. *N. fischeri* shows a broad temperature tolerance for vegetative growth between 10 °C and 52 °C. Growth temperature directly affects the alternation of generations of *N. fischeri*. The sexual form (teleomorph) dominates at 24 °C, while the asexual form (anamorph) can be observed at 37 °C, which is named *Aspergillus fischerianus*. The spores also show a high tolerance against high temperatures and can therefore be found viable in food products after heat treatment, like in fruit juices (Beuchat 1986). The genome of *N. fischeri* NRRL181 was sequenced and published in 2008 (Fedorova *et al.*, 2008). The genome consists of 32 Mb, divided between eight chromosomes, encoding about 10400 proteins (Sanchez *et al.*, 2012). Analysis of the sequences shows that the genome contains 46 genes for the biosynthesis of secondary metabolite backbone structures, including 17 PKS and 19 NRPS genes. The genome further contains 10 putative DMATS genes (Khaldi *et al.*, 2010; Sanchez *et al.*, 2012). Some known secondary metabolites produced by *N. fischeri* are, for example, the fumitremorgin-type indole alkaloids fumitremorgin A (**22**), B (**23**), C (**24**) and verruculogen (**25**) (Figure 1.4) (Nielsen *et al.*, 1988). Another known compound is acetylaszonalenin (**26**) (Yin *et al.*, 2009).

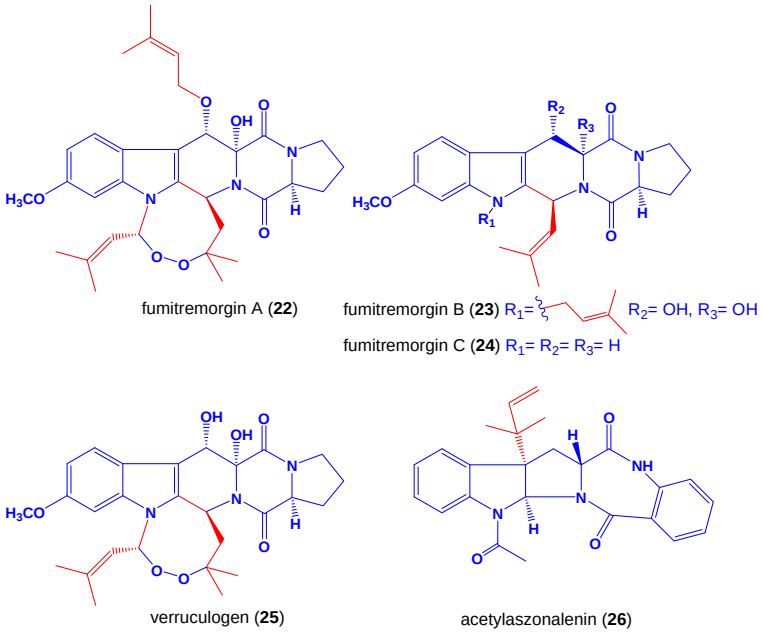


Figure 1.4 Examples of secondary metabolites produced by *Neosartorya fischeri*. Backbone structures are shown in blue and prenyl moieties in red.

1.4 Prenylated aromatic compounds

Prenylated aromatic compounds are hybrid molecules containing an aromatic scaffold that is modified with one or more prenyl moieties derived from prenyl diphosphates. The large structural diversity of aromatic scaffolds and the multitude of possibilities for the attachment of prenyl moieties render these compounds an important class of natural products (Heide 2009). Prenylated aromatic compounds are widely distributed in nature and are produced by bacteria, fungi and plants. Prenylated compounds often gain a specific biological activity different from their non-prenylated precursors (Botta *et al.,* 2005a; El-Seedi *et al.,* 2010; Li 2010). Given the large structural diversity, numerous biological activities have been reported for prenylated aromatic compounds, making these compounds interesting for biological and pharmaceutical research.

By prenylation of a specific compound the lipophilicity is increased, strengthening the interaction of that compound with membranes and proteins (Botta *et al.,* 2005b). This thesis is primarily concerned with prenylated indole alkaloids, prenylated xanthones and benzophenones, as well as prenylated naphthalenes. These compound classes shall be described more in detail.

1.4.1 Prenylated indole alkaloids

Prenylated indole alkaloids are hybrid natural products consisting of an indole or indoline and one or more isoprenoid moieties. In nature, prenylated indole alkaloids are widely distributed in both terrestrial and marine microorganisms and exhibit a huge structural diversity (Li 2010). Besides the indole, which usually derives from L-tryptophan, indole alkaloids often comprise a second amino acid, forming a cyclic dipeptide with a diketopiperazine structure. The formation of cyclic dipeptides is usually catalysed by non-ribosomal peptide synthethases (NRPS), often utilizing L-proline, L-histidine, L-phenylalanine, L-alanine or again L-tryptophan as second amino acid (Li 2010). Several indole alkaloids are biosynthesized by direct prenylation of L-tryptophan, while further modification reactions result in the formation of complex ring systems, like in the biosynthesis of ergot alkaloids or α-cyclopiazonic acid (**20**) (Wallwey & Li 2011; Williams *et al.,* 2000). The prenyl moieties in these biosynthetic pathways originate from prenyl diphosphates, *e. g.* DMAPP, which is connected to the aromatic nucleus by C-1' or C-3', yielding regularly or reversely prenylated compounds, respectively. Important producers of prenylated indole alkaloids are ascomycetous fungi, like members of the genera *Aspergillus*, *Claviceps* and *Penicillium*. Numerous prenylated indole alkaloids show biological and pharmaceutical activities and are in use as drugs. An important source of drugs are the ergot

alkaloids. Ergot alkaloids share the tetracyclic ergoline ring, which is formed by C4-prenylation of L-tryptophan and subsequent modifications. Because of their structural relationship with neurotransmitters, like dopamine or serotonin, ergot alkaloids can interact with several receptors of the central nervous system and therefore exhibit diverse biological activities, such as blood pressure-modulating, uterotonic, control of the secretion of pituitary hormones, migraine-preventive, dopaminergic and neuroleptic activities (de Groot *et al.,* 1998; Haarmann *et al.,* 2009; Schardl *et al.,* 2006). Ergotamine (**27**) and ergometrine (**28**) show vasoconstrictive activity and are in use against migraine and postpartum haemorrhage respectively (Figure 1.5) (Li 2010; Lindel *et al.,* 2012; Wallwey & Li 2011). Known producers of ergot alkaloids among the ascomycetes are fungi of the genera *Claviceps*, *Penicillium* and *Aspergillus* (Schardl *et al.,* 2006). Besides their use in medicine, ergot alkaloids play an important role as food contaminants in history (Lee 2009a; Lee 2009b). In the middle ages, ingestion of ergot-infected grass or grain by animals or humans caused severe epidemics, with symptoms like paranoia, hallucinations and gangrene (Lee 2009a; Schiff 2006).

Further examples of prenylated indole alkaloids with biological activities are tryprostatin A (**9**) and fumitremorgin C (**24**) with inhibitory effects against the breast cancer resistance protein (Jain *et al.,* 2008; Rabindran *et al.,* 2000). Some bis-indolylquinones, like terrequinone A (**18**) from *A. terreus* and *A. nidulans*, show antitumor, antidiabetic and antiretroviral activities (Balibar *et al.,* 2007; Bouhired *et al.,* 2007). Notoamide A (**29**) shows moderate cytotoxic activity against HeLa and L1210 cells (Kato *et al.,* 2007).

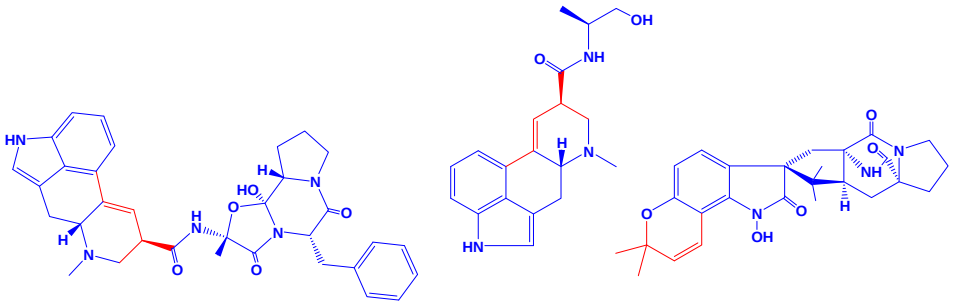


Figure 1.5 Futher examples of prenylated indole alkaloids produced by ascomycetous fungi. Backbone structures are shown in blue and prenyl moieties in red.

1.4.2 Prenylated xanthones

Xanthones are dibenzo-γ-pyrone derivatives produced by higher plants, lichens, fungi and bacteria (El-Seedi *et al.,* 2009; Masters & Bräse 2012; Vieira & Kijjoa 2005). Naturally occurring xanthones carry different substituents at the two benzene rings, thus resulting in a large structural diversity (Sousa & Pinto 2005). The type of substituent is dependent on the

biosynthetic origin and pathway of a given xanthone. Xanthones from plants are hybrid products of shikimate and acetate pathways, and therefore often contain one or more hydroxyl groups. Xanthones from lichens and fungi are polyketides, derived entirely from acetate units, and often contain methyl groups (El-Seedi *et al.,* 2009; El-Seedi *et al.,* 2010; Masters & Bräse 2012). Among the naturally occurring xanthones, prenylated xanthones form an important subgroup (El-Seedi *et al.,* 2009; Pinto & Castanheiro 2009). Due to the large structural diversity and specific substitution patterns, xanthones exhibit numerous biological and pharmaceutical activities. In many cases, a specific biological activity is associated with prenylation of the xanthone skeleton (Pinto *et al.,* 2005). Various biological and pharmaceutical activities, such as antibacterial, antifungal, anti-inflammatory, antioxidant and antitumor, have been reported for prenylated xanthones, which make these compounds attractive for pharmaceutical applications (Castanheiro *et al.,* 2009; El-Seedi *et al.,* 2010; Pinto *et al.,* 2005). A known prenylated xanthone from plants is, for example, guanandin (**30**) from members of the genus *Calophyllum* (Figure 1.6). Guanandin is a dioxygenated xanthone, carrying a regular prenyl moiety in *para*-position to a carbonyl group, and exhibits antihypotensive activity (Gunasekera *et al.,* 1977; Oku *et al.,* 2005). Another example of a C-prenylated xanthone is 2-isoprenyl-1,7-dihydroxy-3-methoxyxanthone (**31**), isolated from several *Garcinia* species (Deachathai *et al.,* 2005; Huang *et al.,* 2001; Rukachaisirikul *et al.,* 2003) with inhibitory effects against human HL60 leukemia cells (Matsumoto *et al.,* 2003). Isocudraniaxanthone B (**32**) is a reversely prenylated, tetraoxygenated xanthone from *Cratoxylum formosa* exhibiting antioxidative activity (Boonnak *et al.,* 2006; Park *et al.,* 2006). Further examples of prenylated xanthones are α-mangostin (**33**) and garcinone E (**34**), also isolated from *Garcinia* species, carrying two and three prenyl moieties, respectively (Na & Xu 2010; Ryu *et al.,* 2011). For α-mangostin, several biological activities have been reported, like inhibition of the aggregation of β-amyloid peptides, which makes this compound interesting for application against Alzheimer's disease (Wang *et al.,* 2012). Furthermore, α-mangostin exhibits anti-inflammatory activity (Chen *et al.,* 2008; Wang *et al.,* 2012). Garcinone E shows antiplasmodial and antiproliferative effects (Han *et al.,* 2008; Lenta *et al.,* 2011).

Besides the C-prenylated xanthones from plants, several xanthones with C- and O-prenylation have been isolated from ascomycetous fungi. Members of the genera *Aspergillus* and *Penicillium* are known producers of prenylated xanthones (Masters & Bräse 2012; Simpson 2012). *A. nidulans* produces the aforementioned variecoxanthone A (**11**), emericellin (**12**), shamixanthone (**13**), epishamixanthone (**14**) and paeciloxanthone (**35**) (Figure 1.6) (Sanchez *et*

al., 2011; Simpson 2012). This thesis is concerned with the biosynthesis of prenylated xanthones from *A. nidulans*.

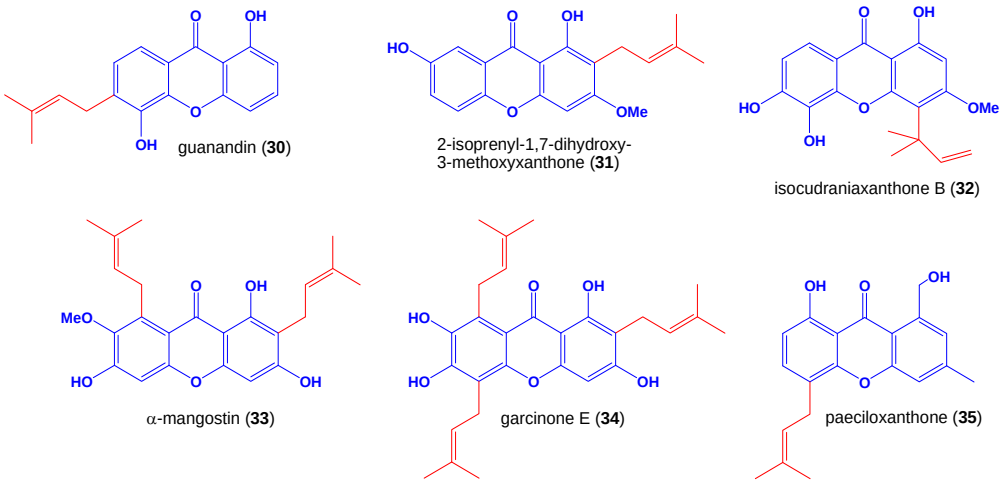


Figure 1.6 Examples of prenylated xanthones with different origins. Backbone structures are shown in blue and prenyl moieties in red.

1.4.3 Prenylated benzophenones

Benzophenones are structurally closely related to xanthones, they also comprise two benzene rings, connected by a carbonyl bridge. By cyclodehydration, the benzophenone can be transformed into the xanthone skeleton. Prenylated benzophenones have been isolated from various plants, like species of the Clusiaceae family. Examples are the guttiferones A (**36**) and C (**37**) isolated from plants of the genera *Garcinia*, *Clusia* and *Symphonia* (Figure 1.7) (Gustafson *et al.,* 1992). These compounds showed interesting biological activities, like inhibitory effects against the HI virus (Fuller *et al.,* 1999). Another example of prenylated benzophenones from *Garcinia* species is garcinol (**38**) with antitumor activity (Saadat & Gupta 2012). Known fungal producers of prenylated benzophenones are *Aspergillus* and *Penicillium* species. Examples of fungal prenylated benzophenones from *Aspergillus* species are arugosin H (**15**), arugosin A (**16**) and B (**17**) (Figure 1.2) (Kralj *et al.,* 2006). Arugosins A and B show moderate cytotoxic and antiproliferative effects against several human cancer cell lines, as well as antimicrobial activity (Greve *et al.,* 2010; Kralj *et al.,* 2006). A threefold prenylated benzophenone, arugosin G (**39**), was isolated from *A. nidulans* (Kralj *et al.,* 2006). An example of prenylated benzophenones from other ascomycetes is arugosin I (**40**) isolated from *Penicillium* ssp. (Lin *et al.,* 2008). Because of the structural resemblance of the prenylated benzophenones and xanthones produced by *A. nidulans*, a common biogenetic origin was postulated (Kralj *et al.,* 2006; Nielsen *et al.,* 2011; Simpson 2012). The prenylation reactions

leading to the diprenylated xanthones and benzophenones from *A. nidulans* are one focus of this thesis.

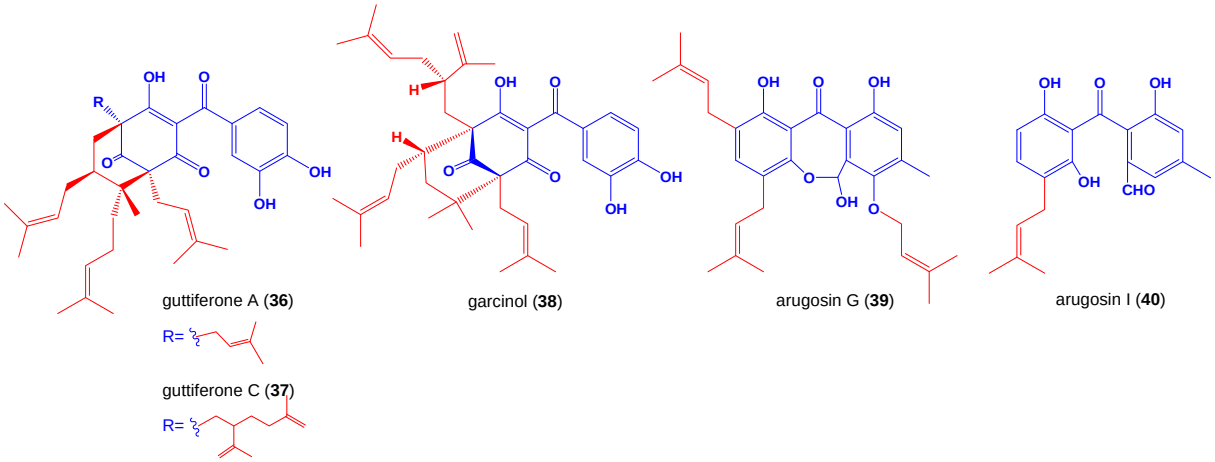


Figure 1.7 Examples of prenylated benzophenones with different origins. Backbone structures are shown in blue and prenyl moieties in red.

1.4.4 Prenylated naphthalene and naphthoquinone derivatives

Naturally occurring prenylated naphthalene derivatives have been isolated exclusively from plants (Hussein *et al.*, 2003; Hussein *et al.*, 2004; Monache *et al.*, 1985) and bacteria (Kumano *et al.*, 2010; Shin-Ya *et al.*, 1990a). An example of a prenylated naphthalene from plants is the C-prenylated vismione E (**41**) (Figure 1.8) from *Cratoxylum* and *Psorospermum* species (Boonnak *et al.*, 2007; Botta *et al.*, 1983; Laphookhieo *et al.*, 2009). Vismione E exhibits antimalarial and antimicrobial activities (Boonnak *et al.*, 2007; Laphookhieo *et al.*, 2009). The threefold prenylated naphthalene derivative adenaflorin C (**42**) has been isolated from *Adenaria floribunda*. Adenaflorin C shows cytotoxic activity against several human cancer cell lines (Hussein *et al.*, 2004). Naphterpin (**43**) is a prenylated naphthoquinone derivative produced by some *Streptomyces* species with antioxidant activities (Seto *et al.*, 1996; Shin-Ya *et al.*, 1990b). Naphterpin is biosynthesized from 1,3,6,8-tetrahydroxynaphthalene and geranyl diphosphate (GPP) (Shin-Ya *et al.*, 1990a).

The compound lapachol (**44**) is a prenylated naphthoquinone, isolated from many plant species (Hussain *et al.*, 2007). Lapachol shows several biological activities, like antitumor, anti-inflammatory and antiviral activities (Epifano *et al.*, 2013; Hussain *et al.*, 2007).

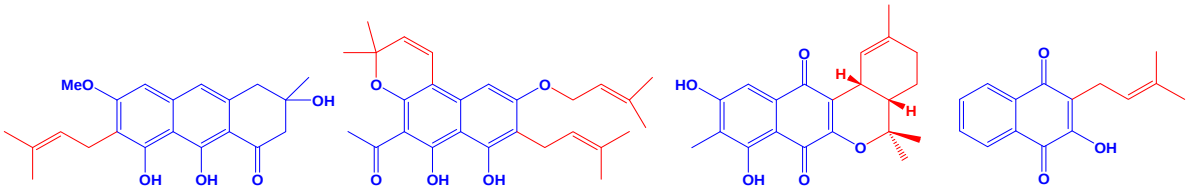


Figure 1.8 Examples of prenylated naphthalene and naphthoquinone derivatives from different origins. Backbone structures are shown in blue and prenyl moieties in red.

1.5 Aromatic prenyltransferases from ascomycetes

Aromatic prenyltransferases catalyse the transfer reactions of prenyl moieties from prenyl diphosphates onto aromatic molecules, thereby contributing to the large structural diversity of natural products. Responsible enzymes from plants, fungi and bacteria belong to different enzyme families or subgroups and have different biochemical properties and primary amino acid sequences (Brandt *et al.*, 2009; Heide 2009; Yazaki *et al.*, 2009; Yu & Li 2012). From ascomycetes, the following three groups of prenyltransferases are known participants in secondary metabolism, which shall be described more in detail.

1.5.1 Prenyltransferases of the UbiA superfamily

Prenyltransferases of the UbiA superfamily are membrane-bound enzymes present in all domains of life with important functions in both primary and secondary metabolism. The eponymous enzyme UbiA from *E. coli* is involved in the biosynthesis of ubiquinone-10 (Melzer & Heide 1994). UbiA catalyses the transfer of a C_{50}-isoprene moiety to 4-hydroxybenzoic acid. Orthologous enzymes have been studied in many different organisms and these enzymes are referred to as prenyltransferases of the UbiA superfamily. The prenyltransferases of this superfamily accept different aromatic compounds as substrates, such as flavonoids, benzoic acids and quinoline derivatives, and prenylate them with isoprene moieties of different carbon chain length (Heide 2009; Stec *et al.*, 2011).

The enzymes SfN8DT, SfG6DT and SfiLDT from *Sophora flavescens*, for example, use flavonoids as substrates (Sasaki *et al.*, 2008; Sasaki *et al.*, 2011). The prenyltransferase LePGT1 from *Lithospermum erythrorizon* catalyses the prenylation of 4-hydroxybenzoic acid in the biosynthesis of shikonin (Yazaki *et al.*, 2002). Common characteristics of the membrane-bound enzymes from the UbiA superfamily are aspartate-rich motifs for substrate binding, as well as the dependency of the catalysed reaction on divalent metal ions (Heide 2009).

1.5.2 Prenyltransferases of the CloQ/NphB group

Prenyltransferases from the CloQ/NphB group are soluble enzymes mainly found in bacteria, but also in some fungi. These enzymes prenylate different aromatic substrates, like hydroxynaphthalenes, quinones, phenols and phenazines (Heide 2009). A member of this group, CloQ from *Streptomyces roseochromogenes* prenylates 4-hydroxyphenylpyruvic acid (Pojer *et al.,* 2003). NphB from *Streptomyces* sp. prenylates 1,3,6,8-tetrahydroxynaphthalene with geranyl diphosphate in the biosynthesis of naphterpin (**43**) (Kumano *et al.,* 2008). Other examples are Fur7 from *Streptomyces* sp. and Fnq26 from *Streptomyces cinnamonensis*, catalysing the geranylation of 2-methoxy-3-methylflaviolin and flaviolin, respectively (Haagen *et al.,* 2007; Kumano *et al.,* 2010). An example of a fungal enzyme is Ptf_{AT} from *A. terreus*, which catalyses the prenylation of 2,7-dihydroxynaphthalene as a non-natural substrate with DMAPP (Haug-Schifferdecker *et al.,* 2010).

One characteristic feature of these enzymes is the tertiary protein structure termed "PT-barrel", consisting of five repetitive αββα-elements. The ten β-strands arrange central as the "barrel" in an antiparallel manner, whereas the α-helices form the solvent-exposed ring around the barrel (Tello *et al.,* 2008). Furthermore, all members of this enzyme group are soluble enzymes that share arginine and lysine-rich motifs for substrate binding and the catalysed reaction is dependent or independent of divalent metal ions (Haug-Schifferdecker *et al.,* 2010; Heide 2009).

1.5.3 Prenyltransferases of the DMATS superfamily

The prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily are soluble proteins found in bacteria and fungi. The first studied member of this superfamily from ascomycetes, DmaW from *Claviceps purpurea*, was identified in 1995 by Tsai and coworkers (Tsai *et al.,* 1995). DmaW and orthologous enzymes from other ascomycetes catalyse the C4-prenylation of L-tryptophan with DMAPP in the biosynthesis of ergot alkaloids and function as a 4-dimethylallyltryptophan synthase (4-DMATS) (Unsöld & Li 2005). With the availability of increasing numbers of genome sequences from ascomycetous fungi, the identification of homologous genes was accelerated (Li 2009). Genes with sequence similarity to *dmaW* are referred to as members of the DMATS superfamily. A large number of enzymes from the DMATS superfamily identified in the last years play an important role in the biosynthesis of secondary metabolites from ascomycetes (Steffan *et al.,* 2009; Yu & Li 2012). The majority of these enzymes catalyse the prenylation of tryptophan derivatives or tryptophan-containing cyclic dipeptides, which were therefore also referred to as indole prenyltransferases several

years ago (Steffan *et al.,* 2009). Until now, prenyltransferases catalysing the prenylation of each position of the indole ring have been identified from different ascomycetes and bacteria (Figure 1.9), distinguishing this enzyme superfamily as a valuable toolbox for the production of prenylated indole derivatives (Pockrandt & Li 2013; Yu & Li 2012). The enzyme FtmPT2 catalyses the N1-prenylation of 12,13-dihydroxyfumitremorgin in the biosynthesis of fumitremorgins from *A. fumigatus*, resulting in the formation of fumitremorgin B (Grundmann *et al.,* 2008). FtmPT1 catalyses the C2-prenylation of brevianamide F in the same pathway (Grundmann & Li 2005). AnaPT catalyses the reverse C3α-prenylation of (*R*)-benzodiazepinedione in the biosynthesis of acetylaszonalenin from *N. fischeri* (Yin et al., 2009). FgaPT2 from *A. fumigatus* catalyses a C4-prenylation, and 5-DMATS from *Aspergillus clavatus* the C5-prenylation of tryptophan (Unsöld & Li 2005; Yu *et al.,* 2012). IptA from *Streptomyces* sp. catalyses a C6-prenylation, and 7-DMATS from *A. fumigatus* the C7-prenylation of tryptophan (Kremer *et al.,* 2007; Takahashi *et al.,* 2010).

With increasing numbers of biochemically characterised members of the DMATS superfamily, enzymes that accept other substrates than indole derivatives have also been identified. SirD from *Leptosphaeria maculans* for example, catalyses the O-prenylation of tyrosine (Figure 1.10) in the biosynthesis of sirodesmin PL (Kremer & Li 2010). Biochemical investigation of SirD showed that the enzyme also catalyses O- and N-prenylations of phenylalanine or tyrosine derivatives. Interestingly, SirD also catalyses the C7-prenylation of L-tryptophan and indole derivatives (Zou *et al.,* 2011). This relaxed substrate specificity regarding the acceptance of aromatic substrates is one characteristic attribute of the DMATS prenyltransferases. For example, enzymes that accept tryptophan usually also accept tryptophan-containing cyclic dipeptides or other aromatic compounds as substrates (Li 2009). On the other hand, a strict regioselectivity of the prenylation position is usually observed (Li 2009). Later, enzymes that accept substrates of non-amino acid origin have also been identified (Figure 1.10). The putative prenyltransferases XptA and XptB from *A. nidulans* were reported to accept polyketide-derived xanthone compounds as substrates, but none of both enzymes has been cloned and characterised biochemically thus far (Sanchez *et al.,* 2011). The enzyme VrtC from *Penicillium aethiopicum* was found to catalyse the geranylation of a polyketide-derived naphthacenedione, transferring a C_{10}-unit from GPP (Chooi *et al.,* 2010). In this work, a DMATS prenyltransferase was implicated for the first time in catalysing the transfer of a prenyl moiety larger than the C_5-unit DMAPP. Interestingly, VrtC accepted only GPP but not DMAPP as prenyl donor.

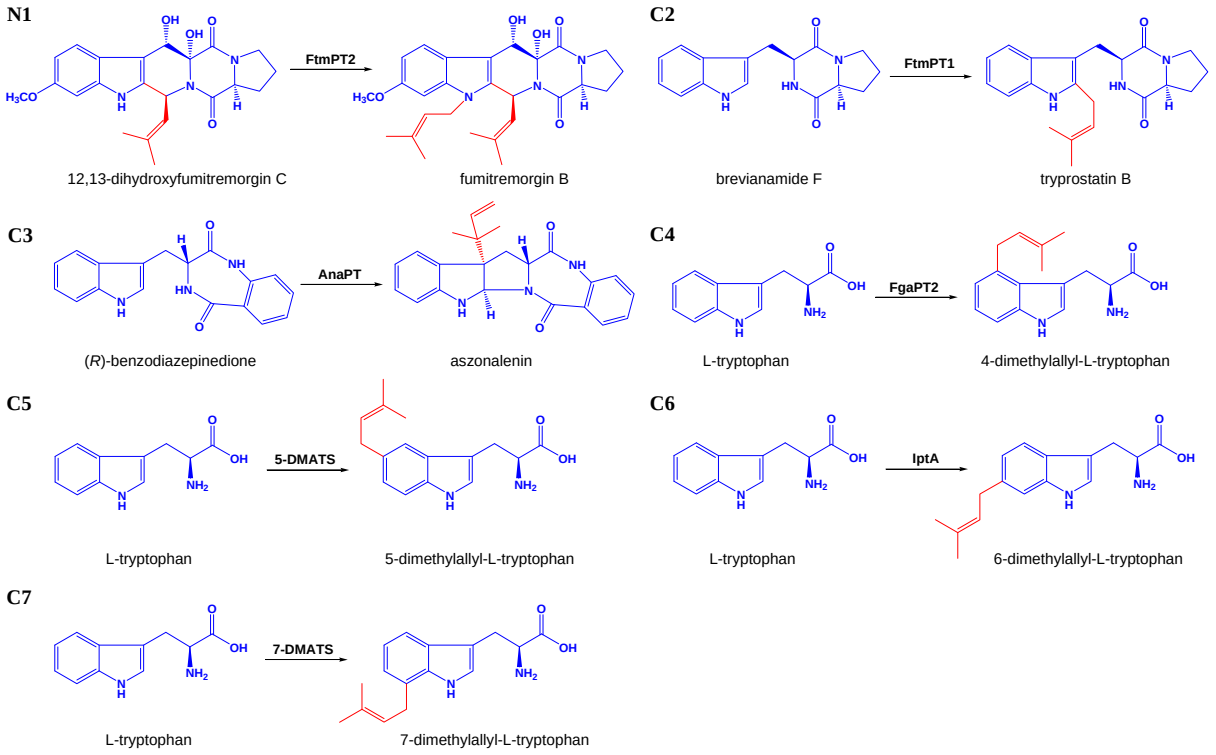


Figure 1.9 Examples of DMATS prenyltransferases and catalysed prenylation reactions at different positions of the indole ring in the presence of DMAPP. Backbone structures are shown in blue and prenyl moieties in red.

Orthologous enzymes of VrtC from other fungi, like NscD from *N. fischeri*, were found to catalyse the prenylation of related polyketide-derived anthracenones with DMAPP, but not GPP (Chooi *et al.*, 2012; Chooi *et al.*, 2013). Some members of the DMATS prenyltransferases accepted also unnatural aromatic substrates, like flavonoids or hydroxynaphthalene derivatives, which are apparently not produced by ascomycetous fungi. 7-DMATS from *A. fumigatus* was shown to prenylate six flavonoids (Yu & Li 2011). Several tested enzymes, like AnaPT and CdpC3PT from *N. fischeri*, as well as CdpNPT and 7-DMATS from *A. fumigatus*, accepted hydroxynaphthalene derivatives as substrates (Yu *et al.*, 2011).

An attempt for explanation of this relaxed substrate specificity was found in the protein structure of the DMATS prenyltransferases. Structural analysis using X-ray crystallography showed that prenyltransferases from the DMATS superfamily exhibit the same tertiary protein structure as the bacterial prenyltransferases from the CloQ/NphB group, while they share no sequence homology on the amino acid level (Jost *et al.*, 2010; Kuzuyama *et al.*, 2005; Metzger *et al.*, 2009; Schuller *et al.*, 2012). Both enzyme groups contain the tertiary protein structure of the PT-barrel, formed by five repetitive αββα-elements, and are therefore gathered in the group of ABBA-prenyltransferases (Bonitz *et al.*, 2011). The large hydrophobic pocket inside the PT-barrel, formed by the antiparallel β-strands, contains the reactive center of the prenyltransferases and minor changes of the amino acid configuration inside this pocket cause

the differences in substrate specificity (Schuller *et al.*, 2012).

Almost all characterised enzymes from the DMATS superfamily accept as prenyl donor exclusively the C_5-unit DMAPP, only the above mentioned VrtC from *Penicillium aethiopicum* was shown to utilize GPP, but not DMAPP, as prenyl donor. An investigation of the prenyl donor specificity showed that some enzymes accept unnatural alkyl diphosphates as donors for the alkylation of aromatic compounds (Liebhold *et al.*, 2012). Interestingly, the regioselectivity for the prenylation position on the accepting substrates can be changed by using different unnatural alkyl donors, expanding the applicability of these enzymes for the production of alkylated aromatic compounds (Liebhold *et al.*, 2013).

Taken together, prenyltransferases of the DMATS superfamily have proven themselves valuable tools for the production of prenylated or alkylated aromatic compounds, not only tryptophan derivatives, but also many other aromatic compounds. Ongoing work with these enzymes increases the possibilities for structural modification, which may be useful for pharmaceutical applications.

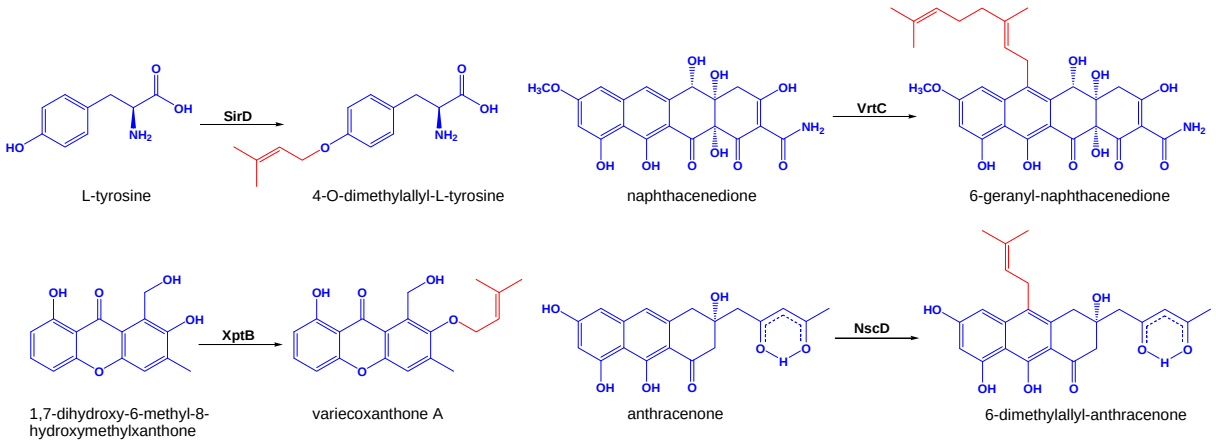


Figure 1.10 Examples of DMATS prenyltransferases that accept substrates other than indole derivatives. Backbone structures are shown in blue and prenyl moieties in red.

1.6 DMATS prenyltransferases from *Aspergillus nidulans* FGSCA4

As mentioned above, the genome of *A. nidulans* FGSCA4 contains seven putative DMATS prenyltransferase genes (Khaldi *et al.*, 2010; Sanchez *et al.*, 2011). With the beginning of the present work in March 2010, only TdiB was biochemically characterised (Table 1.1). The gene *tdiB* (ANID_08514) was identified as an asterriquinone prenyltransferase involved in the biosynthesis of terrequinone A (**18**) (Schneider *et al.*, 2008). Meanwhile, by gene deletion and metabolom analysis of the deletion mutants, Sanchez *et al*. identified the genes ANID_06784 and ANID_12402, encoding two putative xanthone prenyltransferases XptA and XptB, respectively. These enzymes were assigned to the biosynthesis of the diprenylated emericellin

(**12**) and shamixanthones (**13**, **14**) (Figure 1.2) (Sanchez *et al.,* 2011). However, none of the enzymes was cloned and subsequently characterised biochemically. Considering results from earlier feeding experiments with radiolabeled precursors, and the structural relationship of the prenylated compounds, an alternative pathway was postulated for the biosynthesis of the diprenylated xanthones in *A. nidulans* (Nielsen *et al.,* 2011; Simpson 2012). The discrepancy mentioned by Simpson refers to the biosynthesis of the structurally closely related prenylated benzophenones arugosin H (**15**), A (**16**) and B (**17**) from *A. nidulans*. In the alternative pathway, the prenylated xanthones are synthesized *via* non-prenylated benzophenone intermediates, which would be the substrates for the prenyltransferases XptA and XptB identified by Sanchez *et al*. (2011). However, the substrate specificity of the putative xanthone prenyltransferases remained to be resolved and should be investigated in this thesis.

Table 1.1 Genes encoding putative DMATS prenyltransferases from *Aspergillus nidulans* FGSCA4 (GenBank, AspGD).

locus tag	gene	accession in GenBank	exon, (intron) [bp]	deduced protein [aa]	amino acid sequence identity	biosynthetic pathway
ANID_06784	*xptA*	bp 3102076-3103468 of BN001301	210, (52), 1131	446	29% VrtC (*P. aethiopicum*) (Chooi *et al.,* 2010)	shamixanthones (Sanchez *et al.,* 2011)
ANID_08514	*tdiB*	bp 735415-736807 of BN001305.1	624, (64), 492, (69), 144	419	23% 7-DMATS (*A. fumigatus*) (Kremer *et al.,* 2007)	terrequinone A (Schneider *et al.,* 2008)
ANID_10289	-	bp 3570138-3571539 of BN001307.1	1187, (52), 163	449	40% BrePT (*A. versicolor*) (Yin *et al.,* 2013a)	unknown
ANID_11080	*nptA*	bp 634367-632992 of BN001305.1	1190, (71), 115	434	37% BrePT (*A. versicolor*) (Yin *et al.,* 2013a)	nidulanin A (Andersen *et al.,* 2013)
ANID_11194	-	bp 20154-21606 of BN001306.1	270, (58), 354, (39) 732	451	28% VrtC (*P. aethiopicum*) (Chooi *et al.,* 2010)	unknown
ANID_11202	-	bp 18452-19791 of BN001306.1	1140, (74), 126	421	33% CdpC2PT (*N. fischeri*) (Mundt & Li 2013)	unknown
ANID_12402	*xptB*	bp 445323-446753 of BN001302.1	204, (54), 1173	458	27% FgaPT2 (*A. fumigatus*) (Unsöld & Li 2005)	shamixanthones (Sanchez *et al.,* 2011)

Using gene deletion and gene coexpression studies, the gene ANID_11080 (*nptA*) was implicated in the biosynthesis of the prenylated tetracyclopeptide nidulanin A (Andersen *et al.,* 2013). In this biosynthetic pathway, NptA catalyses the reverse N-prenylation of L-kynurenine within the cyclic tetrapeptide. In this thesis, works were carried out with the genes *xptA*, *xptB*, ANID_10289 and ANID_11080 (*nptA*).

1.7 DMATS prenyltransferases from *Aspergillus oryzae* DSM1147

The genome of *A. oryzae* RIB40 contains eight putative DMATS prenyltransferase genes (Khaldi *et al.,* 2010). With the start of this thesis, three of the eight genes were assigned to reactions in the biosynthesis of known or unknown prenylated aromatic compounds (Table 1.2). The gene AO090011000738 was found to encode an orthologous of AtmD in the aflatrem (**21**) biosynthesis in *A. flavus* NRRL3357 (Nicholson *et al.,* 2009). The gene product from AO090120000023 was characterised as a tryptophan-containing cyclic dipeptide prenyltransferase cTrpPT, with preference for cyclo-L-trp-L-trp, shortly before the start of this thesis, but no natural substrate is known for cTrpPT (Zou *et al.,* 2010). The gene AO090026000002 encodes an orthologous of CpaD, a prenyltransferase from *A. oryzae* NBRC 4177 involved in the biosynthesis of α-cyclopiazonic acid (**20**) (Kato *et al.,* 2011; Liu & Walsh 2009). In the present thesis, works were carried out with the unknown putative prenyltransferase genes AO090102000322, AO090701000600 and AO090020000527 from *A. oryzae* strain DSM1147.

Table 1.2 Genes encoding putative DMATS prenyltransferases from *Aspergillus oryzae* RIB40 (GenBank, AspGD).

locus tag	gene	accession in GenBank	exon, (intron) [bp]	deduced protein [aa]	amino acid sequence identity	biosynthetic pathway
AO090102000322	-	bp 868715-870055 of AP007162	1341	446	28% VrtC (*P. aethiopicum*) (Chooi *et al.,* 2010)	unknown
AO090011000738	*atmD*	bp 1889622-1890782 of AP007171.1	1161	386	33% 7-DMATS (*A. fumigatus*) (Kremer *et al.,* 2007)	aflatrem (Nicholson *et al.,* 2009)
AO090020000527	-	bp 1351450-1352767 of AP007167	1124, (49), 145	422	38% CdpNPT (*A. fumigatus*)' (Yin *et al.,* 2007)	unknown
AO090120000023	*cTrpPT* (Zou *et al.,* 2010)	bp 47468-48655 of AP007166.1	1188	395	36% CdpNPT (*A. fumigatus*) (Yin *et al.,* 2007)	unknown
AO090010000082	-	bp 195401-196916 of AP007175.1	27, (46), 1120, (46), 41, (43), 129	438	23% FgaPT2 (*A. fumigatus*) (Unsöld & Li 2005)	unknown
AO090701000600	-	bp 1579931-1581097 of AP007164	1167	388	31% 7-DMATS (*A. fumigatus*) (Kremer *et al.,* 2007)	unknown
AO090026000002	*cpaD*	bp 5192-6569 of AP007159.1	1154, (64), 160	437	45% FgaPT2 (*A. fumigatus*) (Unsöld & Li 2005)	cyclopiazonic acid (Kato *et al.,* 2011; Liu & Walsh 2009)
AO090005001079	-	bp 2863633-2864992 of AP007151.1	1151, (52), 142	430	52% FgaPT2 (*A. fumigatus*) (Unsöld & Li 2005)	unknown

1.8 DMATS prenyltransferases from *Neosartorya fischeri* NRRL181

In the genome of *N. fischeri* NRRL181 ten putative DMATS prenyltransferase genes were identified (Khaldi *et al.,* 2010; Sanchez *et al.,* 2012). Nine genes have been assigned with functions in the biosynthesis of secondary metabolites (Table 1.3). The genes *ftmPT1*, *ftmPT2*

and *ftmPT3* were shown as the prenyltransferases involved in the biosynthesis of fumitremorgins and verruculogen (Grundmann *et al.,* 2008; Grundmann & Li 2005; Mundt *et al.,* 2012). The cyclic dipeptide prenyltransferases CdpC3PT and CdpNPT were characterised biochemically, but no natural substrates are known for both enzymes (Yin *et al.,* 2007; Yin *et al.,* 2010). For the cyclic dipeptide prenyltransferase CdpC2PT involvement in the biosynthesis of fellutanines was discussed (Mundt & Li 2013). AnaPT is part of the biosynthesis cluster of acetylaszonalenin (Yin *et al.,* 2009).

Table 1.3 Genes encoding putative DMATS prenyltransferases from *Neosartorya fischeri* NRRL181 (GenBank, AspGD).

locus tag	gene	accession in GenBank	exon, (intron) [bp]	deduced protein [aa]	amino acid sequence identity	biosynthetic pathway
NFIA_112230	*nscD*	bp 75150-76514 of DS027692 (AAKE03000000)	1365	454	48% VrtC (*P. aethiopicum*) (Chooi *et al.,* 2010)	neosartoricin (Chooi *et al.,* 2013)
NFIA_062330	-	bp 216976-218337 of DS027690 (AAKE03000000)	1362	453	22% FgaPT2 (*A. fumigatus*) (Unsöld & Li 2005)	unknown
NFIA_094090	*cdpNPT* (Yin *et al.,* 2007)	bp 130826-132215 of DS027694 (AAKE03000000)	1214, (67), 109	440	37% cTrpPT (*A. oryzae*) (Zou *et al.,* 2010)	unknown
NFIA_043650	*cdpC2PT* (Mundt & Li 2013)	bp 30953-32343 of DS027684 (AAKE03000020.1)	1220, (53), 118	445	40% BrePT (*A. versicolor*) (Yin *et al.,* 2013a)	unknown
NFIA_064390	*7-dmats*	bp 770120-771591 of DS027690 (AAKE03000000)	1301, (53), 118	472	32% FgaPT2 (*A. fumigatus*) (Unsöld & Li 2005)	astechrome (Kremer *et al.,* 2007; Yin *et al.,* 2013b)
NFIA_093400	*ftmPT3*	bp 6203976-6205399 of NW_001509762	1250, (62), 112	453	35% CdpC3PT (*N. fischeri*) (Yin *et al.,* 2010)	fumitremorgin A (Mundt *et al.,* 2012)
NFIA_074280	*cdpC3PT* (Yin *et al.,* 2010)	bp 569563-570866 of DS027696.1	1172, (53), 100	423	53% CdpNPT (*A. fumigatus*) (Yin *et al.,* 2007)	unknown
NFIA_055300	*anaPT*	bp 2437444-2438808 of NW_001509760	1187, (51), 127	437	33% CdpC2PT (*N. fischeri*) (Mundt & Li 2013)	acetylaszonalenin (Yin *et al.,* 2009)
NFIA_093720	*ftmPT1*	bp 24577-26351 of NW_001509764	1262, (69), 133	464	36% FgaPT2 (*A. fumigatus*) (Unsöld & Li 2005)	fumitremorgins (Grundmann & Li 2005)
NFIA_093760	*ftmPT2*	bp 33832-35013 of NW_001509764	1182	393	36% FgaPT2 (*A. fumigatus*) (Unsöld & Li 2005)	fumitremorgins (Grundmann *et al.,* 2008)

7-DMATS catalyses the C7-prenylation of tryptophan and is involved in the biosynthesis of astechrome (Kremer *et al.,* 2007; Yin *et al.,* 2013b). NscD catalyses the prenylation of an anthracenone substrate in the biosynthesis of neosartoricin (Chooi *et al.,* 2013). The gene NFIA_062330 with unknown function remains to be characterised. The specificity of several DMATS prenyltransferases towards different prenyl donors was investigated in this thesis, including the enzymes CdpC3PT and AnaPT from *N. fischeri* NRRL181.

2 Aims of this thesis

Investigation of the gene functions of four putative DMATS prenyltransferase genes from *Aspergillus nidulans* FGSCA4

In the released genome sequence of *Aspergillus nidulans* FGSCA4 seven putative DMATS prenyltransferase genes were identified (Table 1.1) (Khaldi *et al.,* 2010; Sanchez *et al.,* 2011). However, functional proof on the basis of experiments with purified recombinant enzymes and thoroughly biochemical characterisation was only carried out for TdiB, the prenyltransferase involved in the biosynthesis of asterriquinones (Schneider *et al.,* 2008). The genes *xptA* and *xptB* were assigned to the biosynthesis of the diprenylated emericellin and shamixanthones (Sanchez *et al.,* 2011). However, these postulated functions were only deduced from gene deletion studies and not verified by investigation of the substrate specificity of the enzymes *in vitro*. Nonetheless, this was the first report of an involvement of a member of the DMATS superfamily in the biosynthesis of prenylated xanthones, an interesting compound class from pharmaceutical point of view. Analysis of the substrate specificity of the prenyltransferases will provide insights into the usefulness of these enzymes for chemoenzymatic synthesis. Furthermore, when work was started on these enzymes, an ongoing argument was to settle about whether xanthones or rather structurally related benzophenones are the substrates for both prenyltransferases (Nielsen *et al.,* 2011; Simpson 2012). Biochemical investigation with the recombinant prenyltransferases XptA and XptB, *e. g.* incubation with the postulated natural substrates, would clarify this issue.

Later, the putative DMATS prenyltransferase gene ANID_11080 from the nidulanin A biosynthesis pathway, as well as the gene ANID_10289 with unknown function, were also analysed in this thesis.

To characterise the DMATS prenyltransferases from *A. nidulans* mentioned above, the following works were carried out:

- Amplification and cloning of the prenyltransferase genes *xptA*, *xptB*, ANID_10289 and ANID_11080
- Overproduction and purification of the enzymes XptA and XptB in *E. coli*
- Enzyme assays with the putative xanthone prenyltransferases for investigation of substrate specificity

- Isolation of the enzyme products on HPLC and structural elucidation of the enzyme products by NMR and MS analyses
- Determination of the kinetic parameters

Testing the acceptance of prenyl donors by DMATS prenyltransferases from different *Aspergillus* species

The acceptance of a prenyl donor with a chain length larger than that of DMAPP (C_5-unit) by a DMATS prenyltransferase was first described for VrtC from *Penicillium aethiopicum* (Chooi *et al.,* 2010). No acceptance of GPP or FPP was observed with several characterised enzymes from this superfamily by our group (Grundmann *et al.,* 2008; Grundmann & Li 2005; Kremer *et al.,* 2007; Kremer & Li 2010; Zou *et al.,* 2010). Very low product formation was observed only in incubation mixtures of FgaPT2 with L-tryptophan and GPP (Unsöld & Li 2005). However, there are a number of enzymes that have not been tested for an acceptance of GPP or FPP. 7-DMATS and CdpNPT from *A. fumigatus*, as well as CdpC3PT and AnaPT from *N. fischeri*, are known for their relaxed substrate specificity towards their aromatic substrates (Yu *et al.,* 2011; Yu & Li 2011). The possibility to prenylate aromatic compounds with prenyl donors of larger carbon chain would increase the usage of the DMATS prenyltransferases for the production of prenylated compounds. In this thesis, these enzymes were again analysed with different prenyl donors. The enzymes were therefore incubated with their natural or best-accepted substrates in the presence of GPP and FPP. The following works were carried out:

- Enzyme assays with 7-DMATS, CdpNPT, CdpC3PT and AnaPT in the presence of their natural or best-accepted substrates and the prenyl donors GPP and FPP
- Isolation of the enzyme products on HPLC and structural elucidation of the enzyme products by NMR and MS analyses
- Determination of the kinetic parameters

Investigation of the gene functions of three putative DMATS prenyltransferase genes from *Aspergillus oryzae* DSM1147

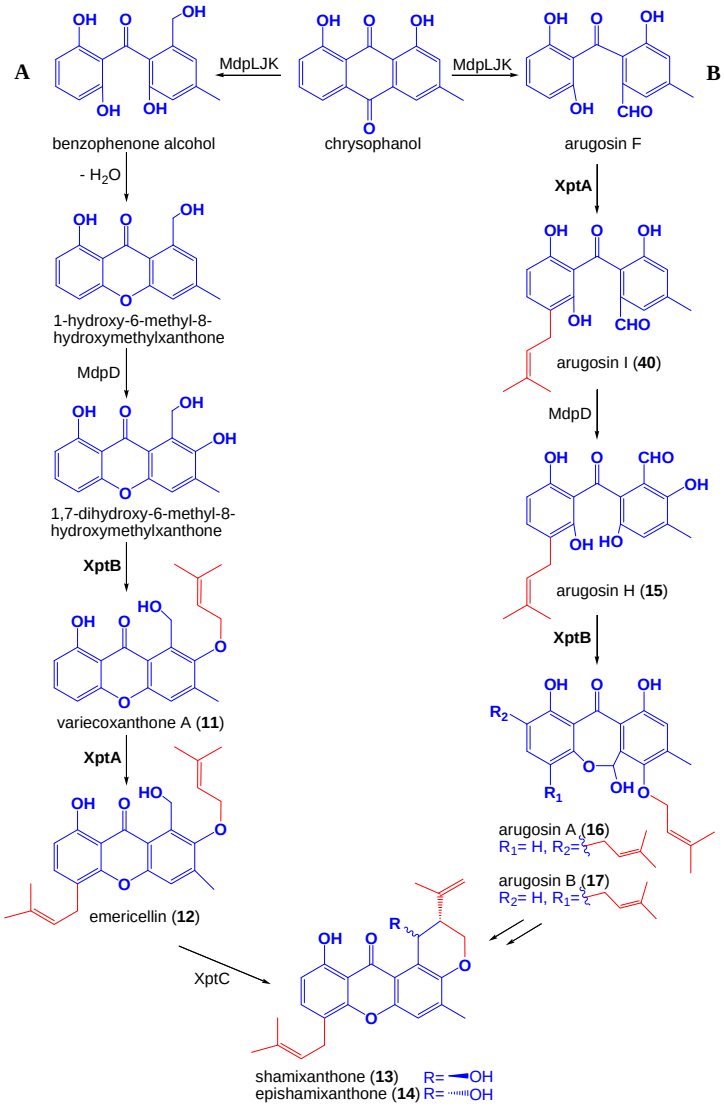
From the genomic sequence of *Aspergillus oryzae* RIB40 eight putative DMATS genes were identified (Khaldi *et al.,* 2010). In this thesis, the three genes AO090102000322, AO090701000600 and AO090020000527 from strain DSM1147 were investigated. These unknown genes should be cloned and overexpressed in *E. coli* and the recombinant proteins investigated biochemically. The following works were carried out:

- Amplification and cloning of the genes AO090102000322, AO090701000600 and AO090020000527
- Overproduction and purification of BAE61387 (AO090102000322) in *E. coli*
- Enzyme assays of BAE61387 with different substrates
- Isolation of the enzyme products on HPLC and structural elucidation of the enzyme products by NMR and MS analyses
- Determination of the kinetic parameters

3 Results and discussion

3.1 Biochemical characterisation of the xanthone prenyltransferase XptB from *Aspergillus nidulans*

Based on gene deletion experiments, the DMATS prenyltransferases XptA and XptB from *A. nidulans* were postulated to be involved in the biosynthesis of diprenylated xanthones (Sanchez *et al.*, 2011). In the proposed biosynthetic pathway by Sanchez *et al.*, the xanthone prenyltransferase B (XptB) catalyses the O-prenylation of 1,7-dihydroxy-6-methyl-8-hydroxymethylxanthone, resulting in the formation of variecoxanthone A (**11**) (Scheme 3.1, A).



Scheme 3.1 Proposed biosynthetic pathways for shamixanthones in *A. nidulans* with (**A**) xanthones and (**B**) benzophenones as substrates for the prenyltransferases XptA and XptB (Simpson 2012). Backbone structures are shown in blue and prenyl moieties in red.

Variecoxanthone A would be further C-prenylated by the xanthone prenyltransferase A (XptA), yielding the diprenylated compound emericellin (**12**), from which eventually the shamixanthones (**13**, **14**) would be formed (Sanchez *et al.*, 2011). This was the first implication of involvement of a member of the DMATS superfamily in the biosynthesis of prenylated xanthones. As mentioned under 1.5.3, the majority of these enzymes are involved in the prenylation of compounds derived from amino acids. However, earlier feeding experiments with radiolabeled precursors suggested that the biosynthesis of the shamixanthones rather proceeds over the formation of benzophenones, which in their turn were postulated to be the substrates of both prenyltransferases (Scheme 3.1, B) (Simpson 2012). In this pathway, XptA would first prenylate arugosin F yielding arugosin I (**40**), from which arugosin H (**15**) would be formed. Arugosin H would then be the substrate of XptB, resulting in the diprenylated arugosins A (**16**) and B (**17**), from which eventually the shamixanthones (**13**, **14**) would be formed. Biochemical investigation of both enzymes regarding their substrate specificity would clarify this discrepancy and provide evidence for their potential for chemoenzymatic synthesis. To characterise these interesting members of the DMATS superfamily and gain an insight in their substrate specificity, both genes were amplified from genomic DNA of *A. nidulans* strain FGSCA4, cloned and expressed in *E. coli*. After purification, the enzyme XptB was incubated with ten xanthones and five benzophenones. XptB accepted four xanthones in the presence of DMAPP, including the proposed natural substrate 1,7-dihydroxy-6-methyl-8-hydroxymethylxanthone. The enzyme products were isolated on HPLC and analysed using NMR and MS. For all accepted xanthones, a regiospecific O-prenylation at OH-7 was identified. None of the tested benzophenone derivatives was accepted by XptB, suggesting that the enzyme is not the prenyltransferase catalysing the prenylation of arugosin H (**15**), which was also a suggested substrate of XptB (Simpson 2012). XptB has been shown a useful enzyme for the regioselective O-prenylation of hydroxylated xanthone derivatives.

For further details of this work, please see publication in section 4.1.
Pockrandt D, Ludwig L, Fan A, König G M, Li S-M (2012) New insights into the biosynthesis of prenylated xanthones: XptB from *Aspergillus nidulans* catalyses an O-prenylation of xanthones. Chembiochem 13, 2764–2771

3.2 Cloning and expression of the xanthone prenyltransferase gene *xptA* from *Aspergillus nidulans*

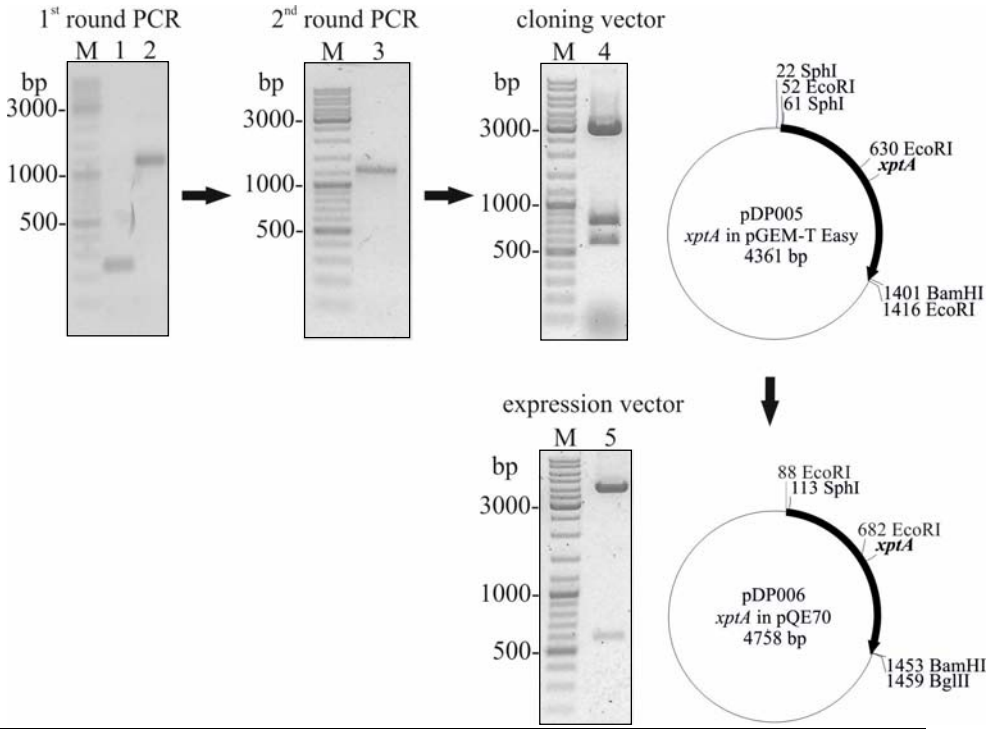
As mentioned in the introduction (Table 1.1), the putative xanthone prenyltransferase gene *xptA* (ANID_06784) is located on chromosome 1 at bp 3102076-3103468 of BN001301 (GenBank). The gene consists of two exons, one small exon of 210 bp at the 5'-end and a large exon of 1131 bp at the 3'-end, separated by an intron of 52 bp. The deduced protein XptA comprises 446 amino acids and shares a sequence similarity of 29% with VrtC from *Penicillium aethiopicum* on the amino acid level. The coding sequence of *xptA* was amplified by PCR from genomic DNA of *A. nidulans* strain FGSCA4 using two-step fusion PCR. The used primer pairs and PCR conditions are shown in table 3.1. In a first round PCR, the single exons were amplified separately (Figure 3.1, lanes 1 and 2). For amplification of the first exon, the primers ANID_06784_*Sph*I and ANID_06784_2 were used. The second exon was amplified using the primers ANID_06784_3 and ANID_06784_*Bam*HI. The primers ANID_06784_2 and ANID_06784_3 carry 20 bp overlapping extensions (underlined bases in table 3.1) at their 5'-ends for subsequent fusion of the PCR products in a second round PCR. In the second round, the PCR products from the first round were mixed in an equimolar ratio, and for amplification of the coding sequence of *xptA*, the primers ANID_06784_*Sph*I and ANID_06784_*Bam*HI were used. The resulting PCR product (Figure 3.1, lane 3) was cloned into pGEM-T Easy vector (Promega) to create the plasmid pDP005, which was controlled by restriction digestion (Figure 3.1, lane 4) and sequenced to confirm sequence integrity.

Table 3.1 Primers used for PCR amplification of *xptA* (ANID_06784).

primer	sequence	annealing temperature	elongation time
ANID_06784_*Sph*I	5'-TACCTCCA**GCATGC**TGAAACACAG-3'	54° C	1:00 min
ANID_06784_2	5'-TTGGGGCCGAGATAGGGGACGACCCAGTGGTAGTGGAAGA-3'		
ANID_06784_3	5'-TCTTCCACTACCACTGGGTCGTCCCCTATCTCGGCCCCAA-3'	54° C	1:30 min
ANID_06784_*Bam*HI	5'-AAGGTGT**GGATCC**CTTGCAGCCCGGTGCC-3'		

Bold letters represent introduced restriction sites for cloning in expression vector, underlined bases were used for fusion of PCR products.

In order to create an expression plasmid for *xptA*, the sequence was excised from pDP005 at the *Sph*I and *Bam*HI sites and subsequently cloned into pQE70 (Qiagen), which has been digested with the same enzymes before, yielding the plasmid pDP006. The plasmid was controlled again by restriction digestion (Figure 3.1, lane 5). For gene expression, pDP006 was transformed into *E. coli* strain SG13009 (Qiagen).



lane	PCR product/plasmid	restriction enzyme	expected fragment size [bp]	approx. observed fragment size [bp]
1	PCR product	-	240	250
2	PCR product	-	1161	1100
3	PCR product	-	1361	1300
4	pDP005	*Eco*RI	2997, 786, 578	3000, 800, 600
5	pDP006	*Eco*RI	4164, 594	4000, 600

Figure 3.1 PCR amplification and cloning of *xptA* in pQE70. Lane M: Gene Ruler DNA ladder.

E. coli SG13009 cells harbouring pDP006 were cultivated in liquid LB medium supplemented with carbenicillin (50 µg ml^{-1}) at 37 °C and 220 rpm to an A_{600nm}=0.6. After addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the cells were cultivated for further 16 h at 22 °C and 220 rpm before harvest. Protein purification of XptA-His_6 with Ni-NTA agarose resin (Qiagen) was carried out according to the manufacturer's instructions. To analyse the purification process, a SDS-PAGE was carried out (Figure 3.2). A protein yield of 0.5 mg XptA-His_6 per litre of bacterial culture was calculated.

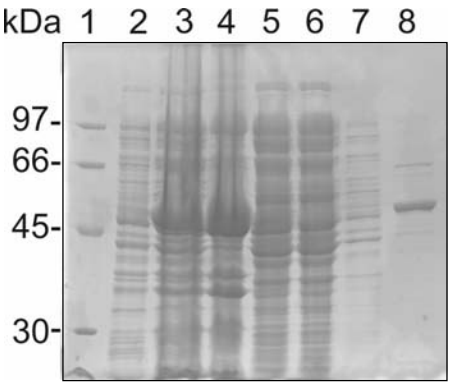


Figure 3.2 SDS-PAGE of the overproduction and purification of XptA-His$_6$. *E. coli* cells SG13009 harbouring pDP006 were cultivated to an A_{600nm}=0.6 and gene expression was induced by addition of 0.1 mM IPTG. The induction time was 16 h at 22 °C and agitation with 220 rpm. Lane 1: molecular mass standard, lane 2: total protein before induction, lane 3: total protein after induction with IPTG, lane 4: insoluble fraction, lane 5: soluble fraction, lane 6: flow-through fraction, lane 7: wash fraction, lane 8: purified XptA-His$_6$.

After purification of the recombinant enzyme, incubations were carried out with the postulated natural substrate variecoxanthone A (**11**) (Sanchez *et al.*, 2011) (Figure 3.3), as well as other potential substrates, including amino acids, tryptophan-containing cyclic dipeptides, hydroxynaphthalene derivatives, xanthones and benzophenones (Table 3.2). Standard incubation mixtures contained 20 µg XptA-His$_6$, 500 µM aromatic substrate, 1 mM DMAPP, 5 mM $CaCl_2$ or $MnCl_2$, 50 mM Tris-HCl buffer pH 7.5, glycerol (1.6% v/v) and dimethyl sulfoxide (DMSO; 2% v/v). The reaction mixtures were incubated at 37 °C for 16 h and extracted twice with two volumes of ethyl acetate. The combined organic phases were evaporated to dryness in a vacuum centrifuge and dissolved in 200 µl methanol for analysis on HPLC. HPLC analysis was carried out as described for XptB enzyme assays (Publication 4.1)

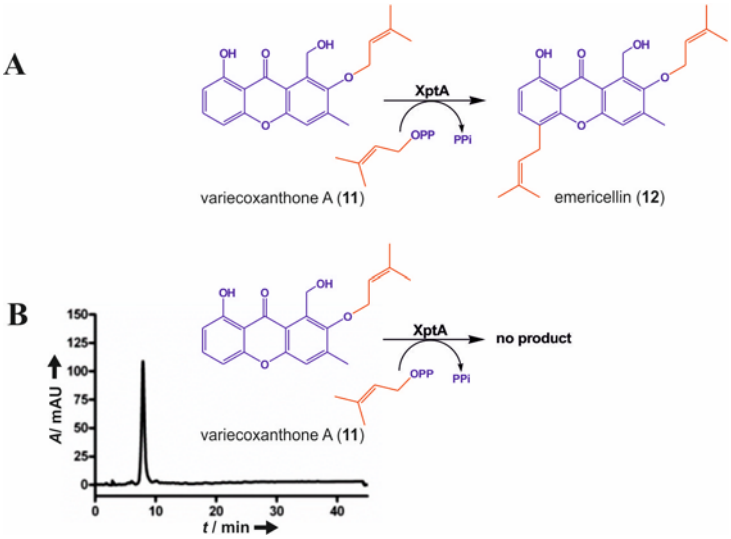


Figure 3.3 (**A**) Postulated C-prenylation of variecoxanthone A (**11**) catalysed by XptA, resulting in emericellin (**12**) (Sanchez *et al.*, 2011). (**B**) HPLC analysis of an enzyme assay with purified XptA-His$_6$ and variecoxanthone A in the presence of DMAPP after incubation for 16 h.

Table 3.2 Aromatic substrates incubated with XptA in the presence of DMAPP.

compound	conversion yield
amino acids	
L-Trp	< 0.05 %
L-abrine	< 0.03 %
L-Tyr	< 0.1 %
D-Tyr	< 0.1 %
tryptophan-containing cyclic dipeptides	
cyclo-L-Trp-L-Leu	< 0.04 %
cyclo-L-Trp-L-Tyr	< 0.02 %
cyclo-L-Trp-L-Trp	< 0.01 %
(*R*)-benzodiazepinedione	< 0.05 %
(*S*)-benzodiazepinedione	< 0.03 %
hydroxynaphthalene derivatives	
1-naphthol	< 0.01 %
1,6-dihydroxynaphthalene	< 0.02 %
1,7-dihydroxynaphthalene	< 0.02 %
2,7-dihydroxynaphthalene	< 0.03 %
xanthone derivatives	
1,3-dihydroxyxanthone	< 0.03 %
1,3,6-trihydroxyxanthone	< 0.01 %
1,3,7-trihydroxyxanthone	< 0.03 %
1,3,6,8-tetrahydroxyxanthone	< 0.02 %
1,7-dihydroxy-6-methylxanthone	< 0.03 %
1,7-dihydroxy-6,8-dimethylxanthone	< 0.03 %
1,7-dihydroxy-5,6,8-trimethylxanthone	< 0.02 %
1,7-dihydroxy-6-methyl-8-formylxanthone	< 0.03 %
1,7-dihydroxy-6-methyl-8-hydroxymethylxanthone	< 0.03 %
variecoxanthone A	< 0.03 %
benzophenone derivatives	
maclurin	< 0.05 %
arugosin H	< 0.03 %
1,5,8-trihydroxybenzophenone	< 0.01 %
5-hydroxy-1,10-dimethoxy-6-carboxybenzophenone	< 0.03 %
5-hydroxy-1,10-dimethoxy-6-carboxylmethylbenzophenone	< 0.03 %

Unfortunately, product formation was observed for none of the tested substrates. The lack of a positive control leaves open whether an active enzyme was purified from *E. coli* cell lysates. Given the low protein yield of 0.5 mg per litre of bacterial culture, and the large amount of overproduced protein observed in the insoluble fraction (Figure 3.2, lane 4), further improvement of the expression conditions should be carried out. The use of lower temperature and IPTG concentrations for gene expression are suggested, in order to lower protein overproduction, which may result in a higher amount of soluble protein.

Further suggested experiments are the use of alternative affinity tags for purification of the recombinant enzyme, like the Strep-tag, speculating that the carboxy-terminal hexahistidine-tag may interfere with protein folding or enzyme activity. Changing the position of the affinity tag from C- to N-terminus may also be tried. The overproduction and purification of XptA-His_6 from other host organisms, like yeast, may also yield an active enzyme.

It may be further speculated that XptA accepts only an unknown substrate, which was neither included in the proposed pathway, nor this study.

3.3 Cloning of the putative DMATS prenyltransferase genes ANID_10289 and ANID_11080 from *Aspergillus nidulans*

The putative prenyltransferase genes ANID_10289 and ANID_11080 from *A. nidulans* were also investigated in this thesis. As mentioned under section 1.6, the gene ANID_10289 is located on chromosome 7 at bp 3570138-3571539 of BN001307.1 (GenBank). The gene consists of two exons, a large exon at the 5'-end with 1187 bp and a small exon with 163 bp at the 3'-end, separated by an intron of 52 bp. The deduced protein CBF86654 comprises 449 amino acids and shares a sequence similarity of 40% with BrePT from *A. versicolor* on the amino acid level. Sequence analysis showed no presence of secondary metabolite backbone genes for the biosynthesis of a possible substrate in the vicinity of ANID_10289. Therefore, no potential substrate could be deduced from sequence analysis. The coding sequence of ANID_10289 was amplified from genomic DNA of *A. nidulans* strain FGSCA4 using two-step fusion PCR. The primers used for PCR amplification are shown in table 3.3. In a first round PCR, the exons were amplified separately, using the primer pairs ANID_10289_*Nco*I and ANID_10289_2 for amplification of the exon at the 5'-end, and the primers ANID_10289_3 and ANID_10289_*Bam*HI for the exon at the 3'-end (Figure 3.4, lanes 1 and 2). The primers ANID_10289_2 and ANID_10289_3 carry 24 bp overlapping extensions at their 5'-ends (underlined bases in tab 3.3) for fusion of the exons in a second round PCR. In the second round, the PCR products from the first round were mixed in an equimolar ratio and the full coding sequence of ANID_10289 was amplified using the primer pairs ANID_10289_*Nco*I and ANID_10289_*Bam*HI. The obtained PCR product (Figure 3.4, lane 3) was cloned into pGEM-T Easy vector creating the plasmid pDP013. pDP013 was controlled using restriction digestion (Figure 3.4, lane 4) and sequenced to confirm sequence integrity.

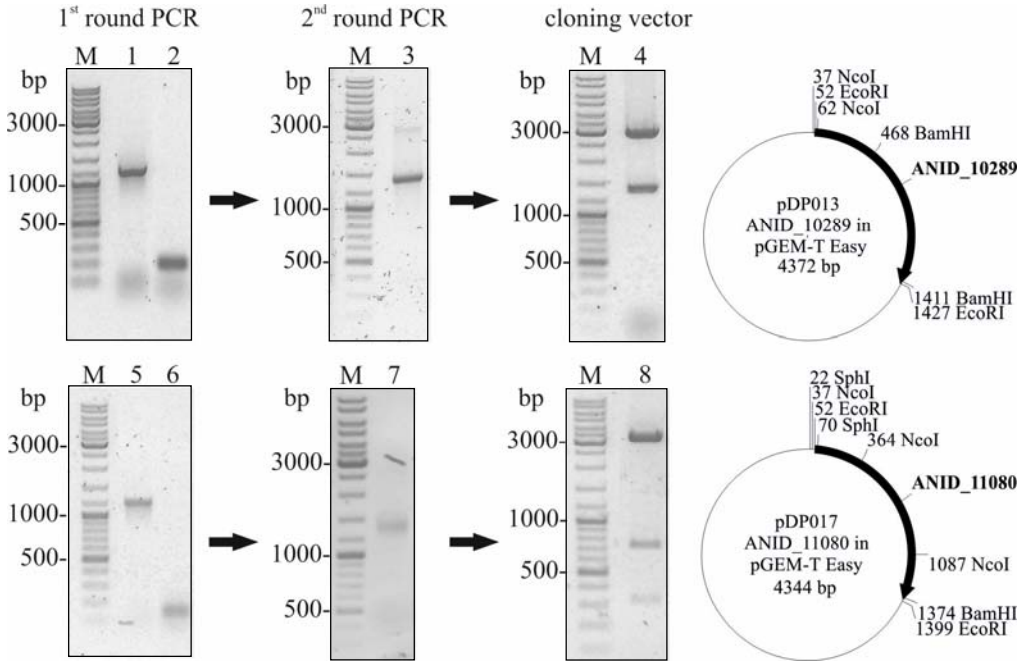
Table 3.3 Primers used for PCR amplification of ANID_10289 and ANID_11080.

primer	sequence	annealing temperature	elongation time
ANID_10289_*Nco*I	5'-AA**CCATGG**CGTCTTTCAGCAACG-3'	64 °C	1:30 min
ANID_10289_2	5'-TCTGACTGATATCGAGATCGGGGCCATCTGCCTTCTCCTG-3'		
ANID_10289_3	5'-CAGCGTGGCAGGAGAAGGCAGATGGCCCCGATCTCGATA-3'	64 °C	1:00 min
ANID_10289_*Bam*HI	5'-C**GGATCC**AGCAGTCCCATCCATCCCG-3'		
ANID_11080_*Sph*I	5'-TTTCAGTCA**GCATGC**TCGCCACAC-3'	50° C	1:30 min
ANID_11080_2	5'-TGGACAGATCGAGGCCAGGATAGAACGACTGAATAGTCTC-3'		
ANID_11080_3	5'-GAGACTATTCAGTCGTTCTATCCTGGCCTCGATCTGTCCA-3'	50° C	1:00 min
ANID_11080_*Bam*HI	5'-ATCAGGCACT**GGATCC**TTTCTCTGGAC-3'		

Bold letters represent introduced restriction sites for cloning in expression vectors, underlined bases were used for fusion of PCR products.

As mentioned in the introduction (section 1.6), the putative prenyltransferase gene ANID_11080 was recently reported to be involved in the biosynthesis of the prenylated cyclotetrapeptide nidulanin A. Using transcriptome analysis, ANID_11080 was found to be

coexpressed with the NRPS gene ANID_01242 (Pes1) located on chromosome 8 at bp 1019461-1038626 of BN001308.1 (GenBank). Gene deletions provided evidence for the role of ANID_01242 and ANID_11080 in the biosynthesis of nidulanin A (Andersen *et al.*, 2013). ANID_11080 is located on chromosome 5 at bp 634367-632992 of BN001305.1 (GenBank). The gene consists of two exons, a large exon at the 5'-end with 1190 bp and a small exon with 115 bp at the 3'-end, separated by an intron of 71 bp. The deduced protein NptA comprises 434 amino acids and shares a sequence similarity of 37% with BrePT from *A. versicolor* on the amino acid level. The coding sequence of ANID_11080 was amplified from genomic DNA of *A. nidulans* strain FGSCA4 using two-step fusion PCR. The primers and PCR conditions are shown in table 3.3.



lane	PCR product/plasmid	restriction enzyme	expected fragment size [bp]	approx. observed fragment size [bp]
1	PCR product	-	1211	1200
2	PCR product	-	187	200
3	PCR product	-	1350	1400
4	pDP013	*Eco*RI	2997, 1375	3000, 1400
5	PCR product	-	1221	1200
6	PCR product	-	148	150
7	PCR product	-	1329	1400
8	pDP017	*Nco*I	3294, 723, 327	3300, 750, 350

Figure 3.4 PCR amplification and cloning of ANID_10289 and ANID_11080 in pGEM-T Easy. Lane M: Gene Ruler DNA ladder.

In a first round PCR, the exons were amplified separately, using the primer pairs ANID_11080_*Sph*I and ANID_11080_2 for the exon at the 5'-end, and the primers ANID_11080_3 and ANID_11080_*Bam*HI for the exon at the 3'-end (Figure 3.4, lanes 5 and 6). The primers ANID_11080_2 and ANID_11080_3 carry 20 bp overlapping extensions at

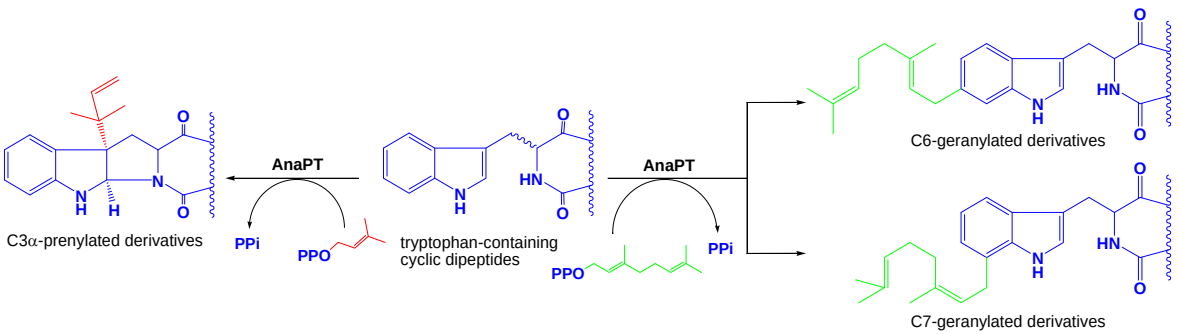
their 5'-ends (underlined bases in tab 3.3) for fusion of the exons in a second round PCR. In the second round, the PCR products from the first round were mixed in an equimolar ratio and the full coding sequence of ANID_11080 was amplified using the primer pairs ANID_11080_*Sph*I and ANID_11080_*Bam*HI. The obtained PCR product (Figure 3.4, lane 7) was cloned into pGEM-T Easy vector creating the plasmid pDP017. pDP017 was controlled using restriction digestion and the expected fragment sizes were observed (Figure 3.4, lane 8). pDP017 was also sequenced to confirm sequence integrity.

The cloning vectors pDP013 and pDP017 were stored at – 20 °C for further work. Due to time constraints, no further works were carried out with both genes.

Suggested experiments are the subcloning of the genes into expression vectors pQE60 and pQE70, respectively. Overproduction in *E. coli* and biochemical investigation of the purified putative prenyltransferases would provide insights into the catalytic activities of these enzymes.

3.4 Specificity of DMATS prenyltransferases from different *Aspergillus* species towards prenyl donors

The DMATS prenyltransferase VrtC from *Penicillium aethiopicum* was reported to catalyse the geranylation of a naphthacenedione derivative, which was the first report of an acceptance of GPP by a DMATS prenyltransferase (Chooi *et al.,* 2010). Later, orthologous enzymes of VrtC were shown to prenylate related anthracenone substrates, utilizing DMAPP rather than GPP as prenyl donor (Chooi *et al.,* 2012), showing the close relationship between DMAPP and GPP-accepting enzymes from the DMATS superfamily. Therefore, the acceptance of prenyl donors by several DMATS prenyltransferases was reinvestigated. 7-DMATS, CdpNPT, CdpC3PT and AnaPT, with known substrate flexibility towards aromatic substrates, were incubated with their natural, or best accepted aromatic substrates in the presence of prenyl donors with longer carbon chain than DMAPP (C_5-unit), such as GPP (C_{10}-unit) and FPP (C_{15}-unit). Initial analysis showed that product formation was only detected in incubation mixtures of AnaPT with (*R*)-benzodiazepinedione in the presence of GPP. Further experiments revealed that six tryptophan-containing cyclic dipeptides were accepted by AnaPT in the presence of GPP. No product formation was detected in incubation mixtures with FPP. Using normal-phase HPLC, two enzyme products were isolated from each of the incubation mixtures. Structural elucidation revealed that products with geranyl moieties at position C-6 or C-7 of the indole ring were obtained. Interestingly, AnaPT catalyses the C3α-prenylation of tryptophan-containing cyclic dipeptides in the presence of DMAPP (Yin *et al.,* 2010). From incubation mixtures of GPP with substrates that contain L-tryptophanyl moieties, a higher yield of C7-geranylated products were obtained, whereas substrates with D-tryptophanyl moieties yielded more C6-geranylated products. So far, AnaPT is the first reported member of the DMATS prenyltransferases that accepts both DMAPP and GPP as prenyl donors, and catalyses different regioselective prenylations with DMAPP and GPP (Scheme 3.2). These findings expand the applicability of the prenyltransferase AnaPT for the production of prenylated products.



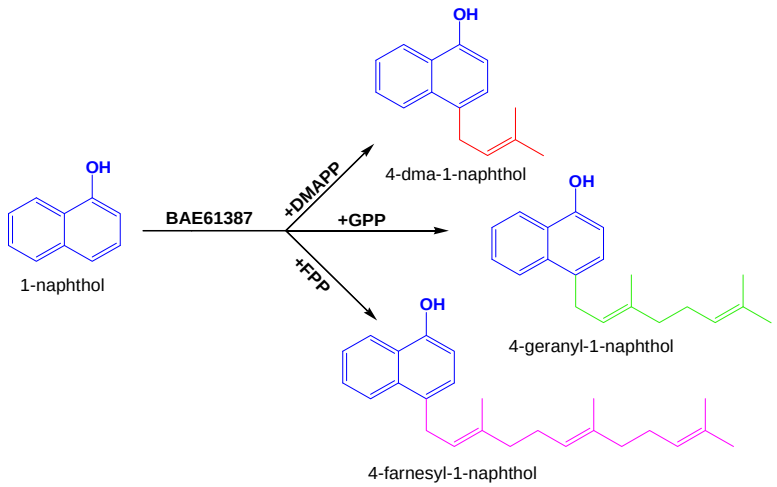
Scheme 3.2 C3α-reverse prenylation with DMAPP, and geranylation at C-6 and C-7 with GPP of tryptophan-containing cyclic dipeptides catalysed by AnaPT. Backbone structures are shown in blue and prenyl moieties in color.

For further details of this work, please see publication in section 4.2. Pockrandt D, Li S-M (2013) Geranylation of cyclic dipeptides by the dimethylallyl transferase AnaPT resulting in a shift of prenylation position on the indole ring. Chembiochem 14, 2023–2028

3.5 Acceptance of DMAPP, GPP and FPP by the prenyltransferase BAE61387 from *Aspergillus oryzae*

The putative prenyltransferase gene AO090102000322 was identified in the genome of *A. oryzae* strain RIB40. Bioinformatic approaches for the identification of a potential natural substrate indicated no inclusion of AO090102000322 in a putative secondary metabolite gene cluster. The gene was amplified by PCR from genomic DNA of *A. oryzae* strain DSM1147, cloned and overexpressed in *E. coli*. After purification, the recombinant protein BAE61387 was incubated with a large number of substrates, which are accepted by many other members of this enzyme superfamily. Initially, product formation was only observed in incubation mixtures with DMAPP and several hydroxynaphthalenes. The acceptance of hydroxynaphthalenes as unnatural substrates in the presence of DMAPP was also shown for other members of the DMATS superfamily (Yu *et al.,* 2011). Interestingly, conversion was also observed after incubation with prenyl donors of longer carbon chain, such as GPP and FPP. The acceptance of both DMAPP and GPP was first shown for AnaPT in this thesis. AnaPT catalyses the C3α-prenylation with DMAPP, and C6- and C7-geranylation with GPP of tryptophan-containing cyclic dipeptides (Pockrandt & Li 2013). However, the acceptance of DMAPP, GPP and FPP as prenyl donors by a member of the DMATS prenyltransferases has not been reported before. Structural elucidation of the obtained enzyme products showed that BAE61387 catalysed regiospecific prenylations with all three prenyl donors. Differing from the results observed for AnaPT, no influence of the prenyl donor chain length on the prenylation position was observed. BAE61387 catalyses the C4-monoprenylation of both 1-naphthol (Scheme 3.3) and 1,7-dihydroxynaphthalene with DMAPP, GPP and FPP. From incubation mixtures with 2,7-dihydroxynaphthalene, C3-monoprenylated products were obtained with all three prenyl donors. Thus, BAE61387 catalyses the prenylation at *para*- or *ortho*-positions of a hydroxyl-group, but not both positions. The conversion yields from incubation mixtures with GPP and FPP were relatively low. Likewise, the kinetic parameters and turnover numbers from reactions of BAE61387 with GPP and FPP suggest that these compounds are not the natural substrates of this enzyme. Alignments of the amino acid sequence of BAE61387 with enzymes from the CloQ/NphB group from bacteria and fungi, which prenylate hydroxynaphthalene derivatives as natural substrates, showed no sequence homology. As mentioned in the introduction, no naturally occurring prenylated hydroxynaphthalene derivatives are known from ascomycetous fungi. More likely, the acceptance of hydroxynaphthalene derivatives by BAE61387 in the presence of DMAPP, GPP and FPP just display the relaxed substrate specificity of the enzyme.

This relaxed substrate specificity, regarding the acceptance of different prenyl donors, makes BAE61387 an interesting enzyme for the production of regiospecific prenylated aromatic compounds.



Scheme 3.3 Regioselective monoprenylation of hydroxynaphthalene derivatives with DMAPP, GPP and FPP catalysed by BAE61387. Backbone structures are shown in blue and prenyl moieties in color.

For further details of this work, please see publication in section 4.3.
Pockrandt D, Sack C, Kosiol T, Li S-M (2014) A promiscuous prenyltransferase from *Aspergillus oryzae* catalyses C-prenylations of hydroxynaphthalenes in the presence of different prenyl donors. Appl Microbiol Biotechnol. 16. DOI: 10.1007/s00253-014-5509-x

3.6 Cloning of the putative DMATS prenyltransferase genes AO090701000600 and AO090020000527 from *Aspergillus oryzae*

The putative prenyltransferase genes AO090701000600 and AO090020000527 with unknown function from *A. oryzae* RIB40 were also investigated as part of this thesis. As mentioned under section 1.7, the putative prenyltransferase gene AO090701000600 consists of a single exon of 1167 bp, located at bp 1579931-1581097 of AP007164 (GenBank). The deduced protein BAE62291 comprises 388 amino acids and shares 31% sequence similarity with 7-DMATS from *A. fumigatus* on the amino acid level. Sequence analysis showed no presence of putative secondary metabolite backbone genes near AO090701000600. Therefore, no potential substrate could be predicted from sequence analysis. The coding sequence of AO090701000600 was amplified by PCR from genomic DNA of *A. oryzae* strain DSM1147 using the primer pair BAE62291_*Sph*I and BAE62291_*Bam*HI (Table 3.4). The obtained PCR product (Figure 3.5, lane 1) was cloned into pGEM-T Easy vector yielding the plasmid pDP011. pDP011 was controlled by restriction digestion (Figure 3.5, lane 2) and sequenced to confirm sequence integrity.

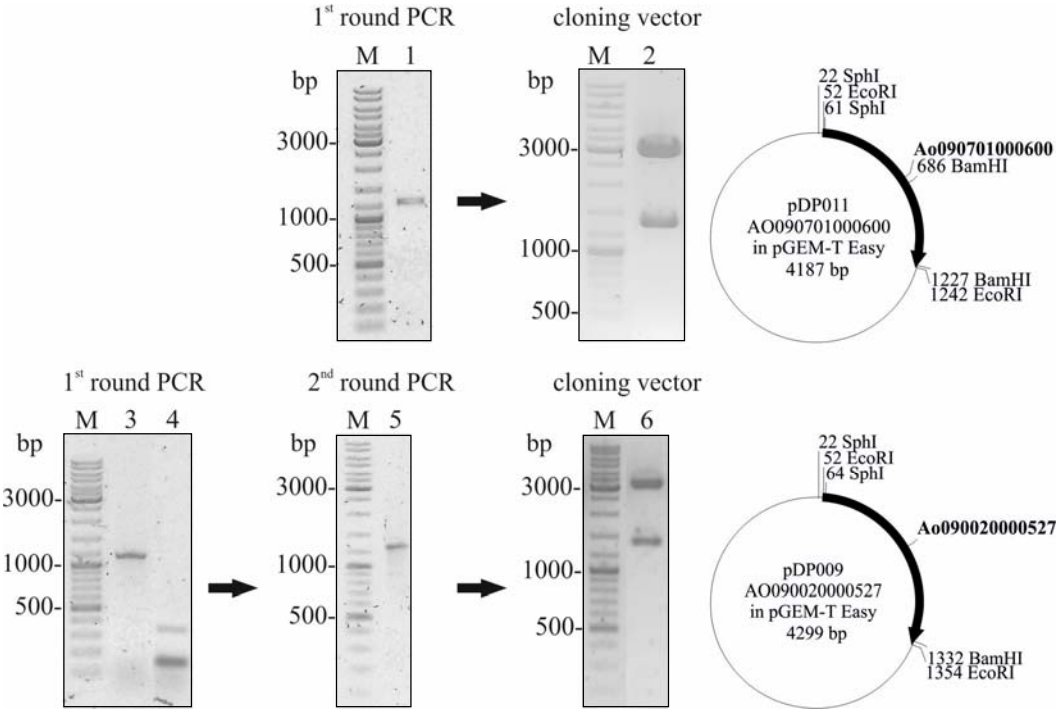
Table 3.4 Primers used for PCR amplification of AO090701000600 (BAE62291) and AO090020000527 (BAE63695).

primer	sequence	annealing temperature	elongation time
BAE62291_*Sph*I	5'-CGC**GCATGC**GAGGCGCTGAATA-3'	51 °C	1:30 min
BAE62291_*Bam*HI	5'-GGCA**GGATCC**GATTGTTTCCG-3'		
BAE63695_*Sph*I	5'-CCAA**GCATGC**TCAACCATAC-3'	49 °C	1:30 min
BAE63695_2	5'-AGATCAACACCAGGGAAGTATTGCAACAGG-3'		
BAE63695_3	5'-CCTGTTGCAATACTTCCCTGGTGTTGATCT-3'	49 °C	1:00 min
BAE63695_*Bam*HI	5'-GTAGACC**GGATCC**GGAGCTGG-3'		

Bold letters represent introduced restriction sites for cloning in expression vectors, underlined bases were used for fusion of PCR products. For convenience, primer designations were given the shorter protein tags.

The second putative prenyltransferase gene AO090020000527 is located at bp 1351450-1352767 of AP007167 (GenBank). The gene consists of two exons, a large exon with 1124 bp at the 5'-end and a small exon with 145 bp at the 3'-end, separated by an intron of 49 bp. The deduced protein BAE63695 comprises 422 amino acids and shares a sequence similarity of 38% with CdpNPT from *A. fumigatus* on the amino acid level. Sequence analysis gave no information about a possible substrate for BAE63695. The coding sequence of AO090020000527 was amplified by two-step fusion PCR from genomic DNA of *A. oryzae* strain DSM1147. The primers are shown in table 3.4. In the first round PCR, both exons were amplified separately, using the primer pairs BAE63695_*Sph*I and BAE63695_2 for amplification of the large exon, and BAE63695_3 and BAE63695_*Bam*HI for amplification of the small exon (Figure 3.5, lanes 3 and 4). The primers BAE63695_2 and BAE63695_3 carry 15 bp overlapping extensions at their 5'-ends for fusion of the PCR products. In the second

round, the PCR products from the first round were mixed in an equimolar ratio and the primers BAE63695_*Sph*I and BAE63695_*Bam*HI were used for amplification of the coding sequence of AO090020000527. The obtained PCR product (Figure 3.5, lane 5) was cloned into pGEM-T Easy vector giving the plasmid pDP009. pDP009 was controlled by restriction digestion (Figure 3.5, lane 6) and sequenced to confirm sequence integrity.



lane	PCR product/plasmid	restriction enzyme	expected fragment size [bp]	approx. observed fragment size [bp]
1	PCR product	-	1179	1250
2	pDP011	*Eco*RI	2997, 1190	3000, 1300
3	PCR product	-	1144	1200
4	PCR product	-	170	150
5	PCR product	-	1284	1300
6	pDP009	*Eco*RI	2997, 1302	3000, 1400

Figure 3.5 PCR amplification and cloning of AO090701000600 and AO090020000527 in pGEM-T Easy. Lane M: Gene Ruler DNA ladder.

The cloning vectors pDP011 and pDP009 were stored at – 20 °C for further work. Due to time constraints, no further works were carried out with both genes.

Suggested experiments are subcloning of the genes into expression vector pQE70. Overproduction in *E. coli* and biochemical investigation of the purified putative prenyltransferases would provide insights in the usefulness of these enzymes for production of prenylated aromatic compounds.

4 Publications

4.1 New insights into the biosynthesis of prenylated xanthones: XptB from *Aspergillus nidulans* catalyses an O-prenylation of xanthones

DOI: 10.1002/cbic.201200545

New Insights into the Biosynthesis of Prenylated Xanthones: Xptb from *Aspergillus nidulans* Catalyses an O-Prenylation of Xanthones

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Gene-inactivation experiments have indicated that the putative prenyltransferase XptB from *Aspergillus nidulans* was likely to be responsible for the prenylation of 1,7-dihydroxy-6-methyl-8-hydroxymethylxanthone. Recently, it was suggested that this enzyme might also accept as substrate the benzophenone arugosin H, which is assumed to be a precursor of prenylated xanthones. In this study, five benzophenones and ten xanthones were incubated with purified recombinant XptB in the presence of dimethylallyl diphosphate (DMAPP). XptB accepted four xanthones as substrates, including the proposed natural substrate, and catalysed regiospecific O-prenylations at C-7 of the xanthone core. K_m values in the range of 0.081–1.1 mM and turnover numbers (k_{cat}) between 0.02 and 0.5 s^{-1} were determined for the accepted xanthones. The kinetic parameters for DMAPP were found to be 0.024 mM (K_m) and 0.13 s^{-1} (k_{cat}). Arugosin H was not accepted by XptB under the tested conditions. XptB was relatively specific towards its prenyl donor and did not accept geranyl or farnesyl diphosphate as substrate. Mn^{2+} and Co^{2+} strongly enhanced XptB activity (up to eightfold); this has not been reported before for prenyltransferases of the DMATS superfamily.

Introduction

Xanthones are dibenzo-γ-pyrone derivatives produced by higher plants, lichens and fungi.[1–3] Naturally occurring xanthones contain different substituents on the two benzene rings, thus resulting in large structural diversity.[4] These substituents are strongly dependent on their biosynthetic origins and modification reactions. For example, xanthones from plants are hybrid molecules of shikimate and acetate pathways and therefore often carry hydroxy groups. Xanthones from lichens and fungi are polyketides derived entirely from acetate units, and contain in many cases a methyl group.[2,3,5] Among naturally occurring xanthones, prenylated derivatives form an important subgroup.[2,6]

Because of their specific substitution patterns, xanthones exhibit diverse biological and pharmaceutical activities. In many cases, activity is associated with prenylation of the xanthone skeleton.[7] Various biological and pharmacological activities, such as antibacterial, antifungal, anti-inflammatory, antioxidant and antitumor, have been reported for prenylated xanthones, which make these compounds attractive for pharmaceutical applications.[5,7,8]

Ascomycetes fungi, especially of the genera *Aspergillus* and *Penicillium,* are known producers of prenylated xanthones.[3,9] For example, *Aspergillus nidulans* was reported to produce the prenylated xanthones variecoxanthon A (**1**), emericellin (**2**), shamixanthone (**3**) and epishamixanthone (**4**) (Scheme 1).[9,10] In addition to prenylated xanthones, *A. nidulans* and its teleomorph *Emericella nidulans* also produce structurally related prenylated benzophenones, such as arugosins A (**5**), B (**6**), H (**7**) and I (**8**) (Scheme 1).[9,11,12]

Genome-based deletion experiments revealed the presence of genetic information for the biosynthesis of prenylated xanthones in *A. nidulans*.[10] Several enzymes, including two putative prenyltransferases XptA and XptB, were assigned to reaction steps in this pathway. Based on the obtained results, a pathway for the biosynthesis of **3** and its stereoisomer **4** was postulated by Sanchez and co-workers.[10] This proposed pathway has some discrepancies with respect to the number of prenylated benzophenones, such as the arugosins mentioned above, that have been isolated from *A. nidulans*.[11] Furthermore, feeding experiments have shown that prenylated benzophenones are likely precursors for the biosynthesis of the prenylated xanthones.[9,12] Based on the results obtained from isotopic labelling studies and chemical precedents, as well as gene-deletion studies, Simpson has recently revised the postulated pathway (pathway A in Scheme 1) and also suggested an alternative pathway (B in Scheme 1).[9] In both cases, the anthraquinone chrysophanol (**9**) rather than the originally suggested monodictyphenone from emodin is the precursor of the prenylated xanthones. Compound **9** is converted by oxidative cleavage of the quinone ring with the help of enzymes MdpL and MdpJ to a thiolester intermediate (**10**), which serves

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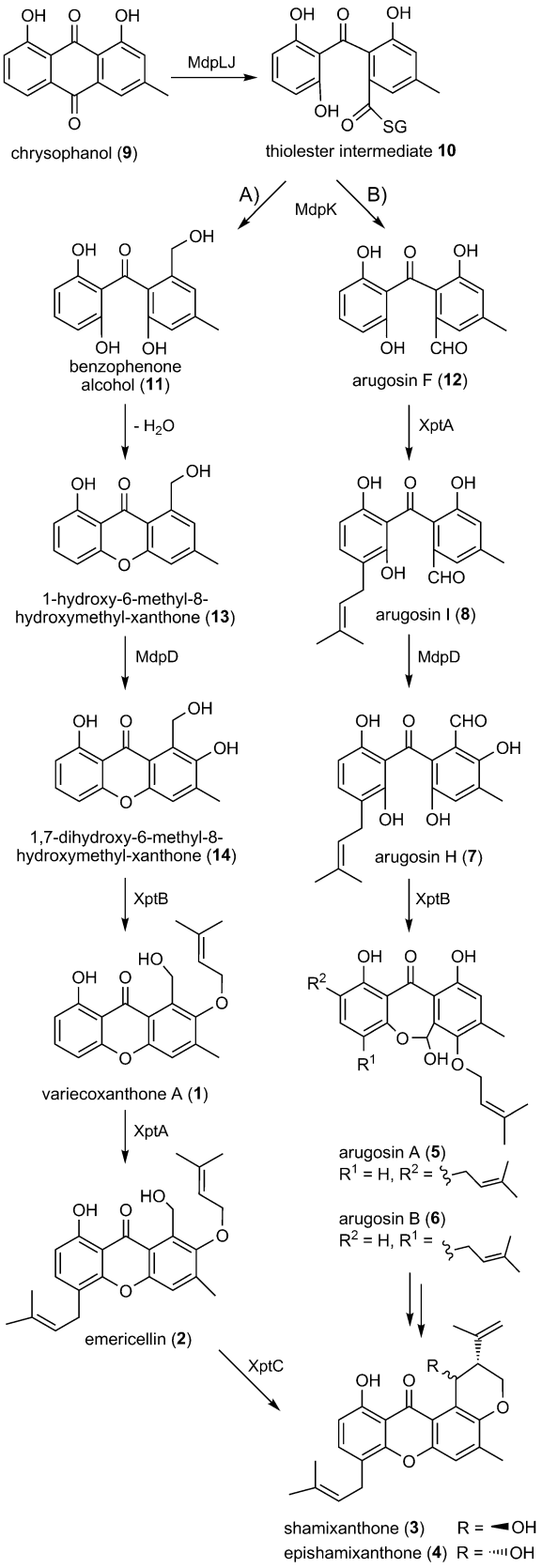
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201200545.



Scheme 1. Proposed biosynthetic pathways of prenylated xanthones in *A. nidulans*, A) with xanthones or B) with benzophenones as substrates for the prenyltransferases XptA and XptB.[9]

as the branch point for the two pathways; it would be reduced by the oxidoreductase MdpK to either a benzophenone alcohol (**11**; pathway A) or the aldehyde, arugosin F (**12**; pathway B). Compound **11** is converted to 1-hydroxy-6-methyl-8-hydroxymethylxanthone (**13**) by elimination of a water molecule, and further hydroxylated by the monooxygenase MdpD to yield 1,7-dihydroxy-6-methyl-8-hydroxymethylxanthone (**14**). Gene-deletion experiments indicated that XptB is very likely responsible for the O-prenylation of **14**, thereby resulting in the formation of variecoxanthone A (**1**). This would be C-prenylated by XptA to **2** and subsequently cyclised to **3** and **4** by the putative oxidoreductase XptC.[10] In the alternative pathway (pathway B), **3** and **4** are formed from **9** via **12** and other known prenylated benzophenones (**5**–**8**).[9]

Comparison of the two pathways revealed two significant differences. 1) The substrates of both prenyltransferases XptA and XptB are xanthones in pathway A, but benzophenones in pathway B (Scheme 1); in pathway A prenylations take place after ring closure, and in pathway B they precede closure. 2) The order of the two prenylation steps differs between the pathways: in pathway A, O-prenylation catalysed by XptB takes place before C-prenylation by XptA, whereas in pathway B, the XptA reaction precedes that of XptB. To provide more details on the biosynthesis of prenylated xanthones, we cloned and overexpressed *xptB* in *Escherichia coli* and incubated the purified protein with ten xanthones and five benzophenones, including the proposed substrates **14** in pathway A and arugosin H (**7**) in pathway B (Scheme 1). Here, we report the acceptance of these substances by XptB and the identification of the enzyme products. The involvement of prenylated benzophenones in the biosynthesis of prenylated xanthones is also discussed.

Results

Sequence analysis and cloning of *xptB*

The putative prenyltransferase gene *xptB* is located on chromosome II of *A. nidulans* and is not clustered with any obvious secondary metabolite genes.[10] BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequence comparison with known members of the DMATS superfamily from our previous works allowed us to identify a putative gene with 29% amino acid sequence identity to SirD from *Leptosphaeria maculans*[13] and 27% identity to FgaPT2 from *Aspergillus fumigatus*.[14] The putative *xptB* gene consists of two exons: a small exon (204 bp) at the 5′-end (bp 445323–445526; GenBank accession BN001302.1) and a larger one (1173 bp) at the 3′-end (bp 445581 to 446753). These are separated by a 54 bp intron. The deduced protein, XptB, comprises 458 amino acids with a calculated molecular mass of 51.9 kDa. The coding region of *xptB* was amplified from genomic DNA of *A. nidulans* FGSC A4 by two-round fusion PCR with four oligonucleotide primers (see the Experimental Section).[15] The obtained PCR fragment was cloned into vector pGEM-T Easy, and then subcloned into the expression vector pQE60 at the NcoI and BamHI sites to create the expression construct pDP008.

Overexpression and purification of XptB-His$_6$

For gene expression, *E. coli* cells harbouring pDP008 were cultivated at 37 °C and induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. The recombinant protein was purified on Ni-NTA agarose resin, and a major protein band above the 45 kDa marker band was detected by SDS-PAGE (Figure S1 in the Supporting Information); this corresponded well with the calculated molecular mass of XptB-His$_6$ (52.9 kDa). The protein yield was 3.5 mg of purified XptB-His$_6$ per litre of culture.

Enzyme activity and substrate specificity of XptB

XptB was assayed with ten xanthones (**14**–**23**; **14** is its proposed natural substrate)[10] and five benzophenones (**7**, **24**–**27**; **7** is another proposed substrate)[9] in the presence of dimethylallyl diphosphate (DMAPP; see Tables 1 and 2). After incubation at 37 °C for 16 h with 20 μg of XptB-His$_6$ in each 200 μL assay, the ethyl acetate extracts of the reaction mixtures were analysed by HPLC. Product formation was clearly detected for four xanthones (Figure 1 and Table 1), with the highest conversion yield (76%) observed for **14**. Three methylated xanthones 1,7-dihydroxy-6-methylxanthone (**19**), 1,7-dihydroxy-6,8-dimethylxanthone (**21**) and 1,7-dihydroxy-5,6,8-trimethylxanthone (**22**) were converted with yields of 23, 25 and 31%, respectively. Product formation in these assays was strictly dependent on the presence of DMAPP and active enzyme. Negative controls with heat-inactivated protein (boiled for 20 min) showed no product peak in the HPLC chromatograms of the incubation mixtures. Geranyl and farnesyl diphosphate were not accepted by XptB as prenyl donors (data not shown). No product formation was detected for other xanthones (**15**–**18**, **20** and **23**; data not shown). In the HPLC chromatogram of the incubation mixture with arugosin H (**7**; Figure 2A), neither the expected products (arugosins A/B, **5**/**6**) nor other products were detected under the tested conditions. Because of the presence of several tautomers,[9,12] **5**/**6** appear as a very broad peak in the HPLC chromatogram (Figure 2B), as observed previously.[12] Negative results were also observed for other tested benzophenones (**24**–**27**; Table 2).

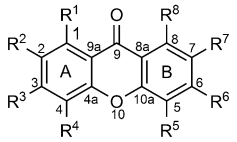
From the obtained results, it can be concluded that a xanthone skeleton is a prerequisite for acceptance by XptB. All the accepted substances bear two hydroxy groups, one at C-1 of ring A and the other at C-7 of ring B (Tables 1 and 2). At the *ortho*-position to C-7, that is, C-6 or C-8, at least one methyl or hydroxymethyl group is attached. It seems that these substituents are also important for XptB enzyme activity. Substances lacking the hydroxy group at C-7 (**15, 16, 18** and **20**) were not accepted by XptB. Negative results were also observed for compounds without the mentioned methyl or hydroxymethyl groups (**17** and **23**).

Because of the previously observed substrate promiscuity of some known prenyltransferases of the DMATS superfamily,[16–18] XptB was also assayed with aromatic amino acids (e.g., L-tryptophan and L-tyrosine) and tryptophan-containing cyclic dipeptides, which are substrates for many characterised enzymes of this class.[13,18–21] HPLC analysis showed that very low conversion (near the detection limit) was only observed for cyclic dipeptides (data not shown).

XptB catalyses regular O-prenylation at the hydroxy group of C-7 of the xanthone ring

For structural elucidation, the enzyme products **1**, **28**, **29** and **30** were isolated by preparative HPLC from the incubation mixtures of XptB with **14**, **19**, **21** and **22**, respectively, and subjected to ^{1}H NMR and MS analyses (Tables 3 and S1). HR-MS confirmed monoprenylation in **1**, **28**, **29** and **30** by detection of the molecular masses (68 Da larger than those of the respective substrates; Table S1). The ^{1}H NMR data of the isolated enzyme products **1**, **28**, **29** and **30** clearly showed the presence of signals for a regular prenyl moiety, each in the range 5.52–5.61 (t, J = 7.2 Hz, H-2′), 4.25–4.64 (d, J = 7.2 Hz, H-1′), 1.71–1.78 (s, 3H-4′) and 1.79–1.82 ppm (s, 3H-5′) (Table 3). Meanwhile, the chemical shifts of H-1′ of the prenyl moiety at 4.25–4.64 ppm proved its attachment to a hetero atom rather than to a C-atom, that is, to a hydroxy group in the isolated

Table 1. Acceptance of xanthones by XptB.

Xanthone									Conversion yield [%]
	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	
1,7-dihydroxy-6-methyl-8-hydroxymethylxanthone (**14**)	OH	H	H	H	H	CH_3	OH	CH_2OH	76
1,3-dihydroxyxanthone (**15**)	OH	H	OH	H	H	H	H	H	< 0.03
1,3,6-trihydroxyxanthone (**16**)	OH	H	OH	H	H	OH	H	H	< 0.02
1,3,7-trihydroxyxanthone (**17**)	OH	H	OH	H	H	H	OH	H	< 0.02
1,3,6,8-tetrahydroxyxanthone (**18**)	OH	H	OH	H	H	OH	H	OH	< 0.03
1,7-dihydroxy-6-methylxanthone (**19**)	OH	H	H	H	H	CH_3	OH	H	23
1-hydroxy-6,8-dimethylxanthone (**20**)	OH	H	H	H	H	CH_3	H	CH_3	< 0.01
1,7-dihydroxy-6,8-dimethylxanthone (**21**)	OH	H	H	H	H	CH_3	OH	CH_3	25
1,7-dihydroxy-5,6,8-trimethylxanthone (**22**)	OH	H	H	H	CH_3	CH_3	OH	CH_3	31
1,7-dihydroxy-6-methyl-8-formylxanthone (**23**)	OH	H	H	H	H	CH_3	OH	CHO	< 0.03

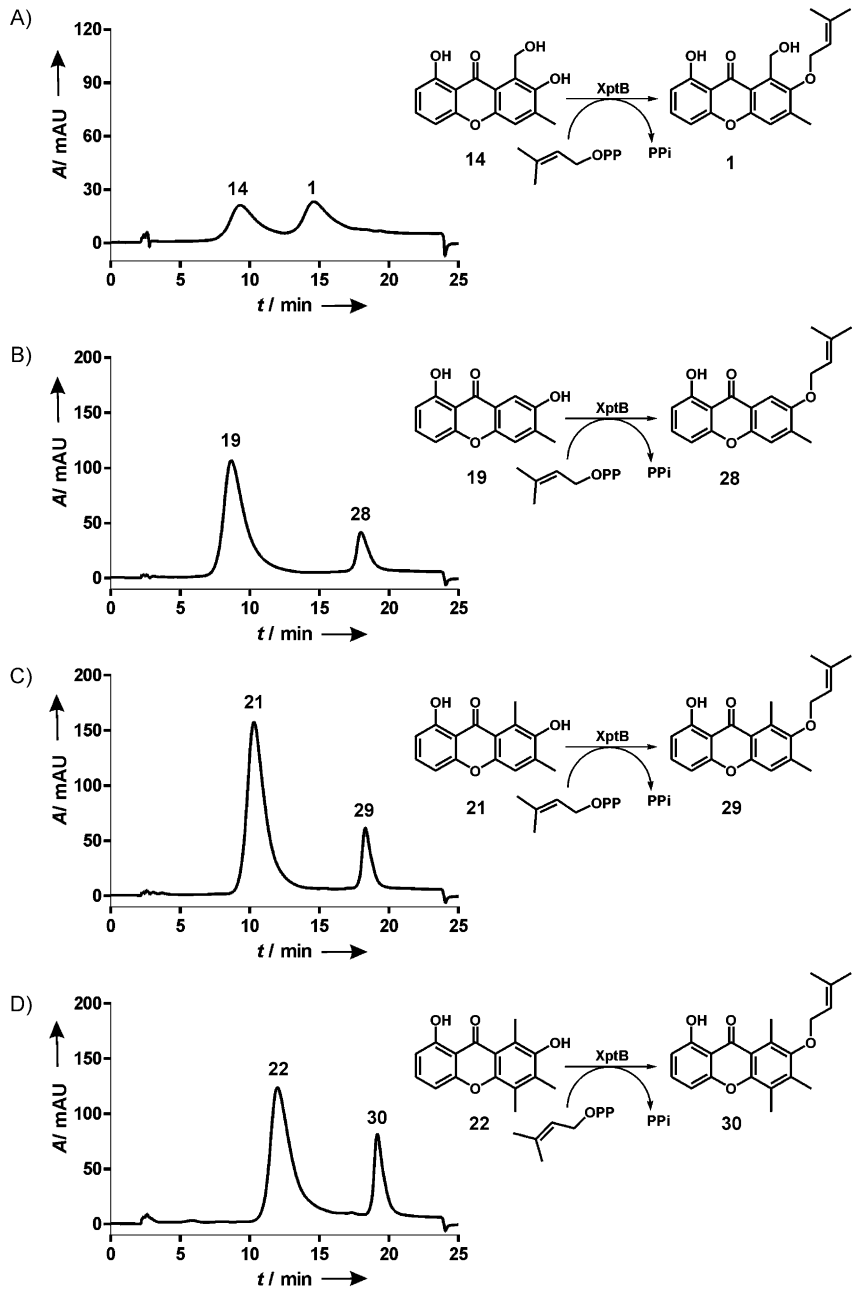


Figure 1. HPLC analysis of enzyme assays with XptB and selected xanthones. Standard incubation mixtures contained 20 μg XptB-His_6, 0.25 mM aromatic substrate, 1 mM DMAPP and 5 mM $MnCl_2$ in 50 mM Tris·HCl, pH 7.5, and were incubated for 16 h at 37 °C. The xanthones were A) **14**, B) **19**, C) **21** and D) **22**.

products. In comparison to those of **14**, **19**, **21** and **22**, the number and coupling patterns of the aromatic protons were not significantly changed in the spectra of **1**, **28**, **29** and **30**, thus indicating that prenylation had also taken place at one of the two OH-groups at C-1 and C-7. The obtained NMR data for **1** are in agreement with those published previously for variecoxanthone A,[10] thus confirming O-prenylation at C-7. In the NOESY spectrum of **29** (Figure S2), interactions between the signals of the two H-1′ protons of the prenyl moiety at 4.29 ppm and those of the two methyl groups at C-6 and C-8 of the xanthone ring at 2.85 and 2.44 ppm were clearly observed, which proves O-prenylation at C-7. Comparison of the NMR data for **28** and **30** with those for the respective substrates (**19** and **22**; Table 3) revealed identical chemical shifts for all the aromatic protons. The chemical shifts of the methyl groups at the aromatic ring were slightly changed, thus also indicating O-prenylation at C-7 rather than C-2. This proved that the hydroxy group at C-7 is the preferred prenylation position for XptB.

Biochemical characterisation of XptB

The reactions of most enzymes of the DMATS superfamily are independent of the presence of metal ions, although Ca^{2+} increases the reaction velocities.[16,22] To test the dependency of XptB activity on metal ions, incubations were carried out with **14** and DMAPP in the presence of diverse divalent ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}. As shown in Figure 3, addition of EDTA (final concentration 5 mM) did not change the enzyme activity significantly. Surprisingly, addition of 5 mM divalent metal ion resulted in a strong increase in the enzyme activity: four- to fivefold in incubation mixtures with Ca^{2+}, Mg^{2+} and Ni^{2+}; in the presence of 5 mM Co^{2+} or Mn^{2+}, the increase in enzyme activity was nearly eightfold. Such phenomena have never been observed for members of the DMATS superfamily. Determination of kinetic parameters was therefore carried out with 5 mM Mn^{2+} in the incubation mixtures.

The kinetic parameters of XptB were determined for all accepted xanthones and for DMAPP (Table 4). Interestingly, the two xanthones **21** and **22** showed K_m values of about 0.08 mM and were better accepted by XptB than its proposed natural substrate **14** ($K_m = 0.327$ mM). Similar phenomena have occasionally been observed for other prenyltransferases.[23] Among the accepted xanthones, the turnover number of **14** ($k_{cat} = 0.43\ s^{-1}$) was threefold higher than those of **21** and **22**. As consequence, XptB showed similar catalytic efficiencies towards **14**, **21** and **22**. Compound **19,** with only one methyl group

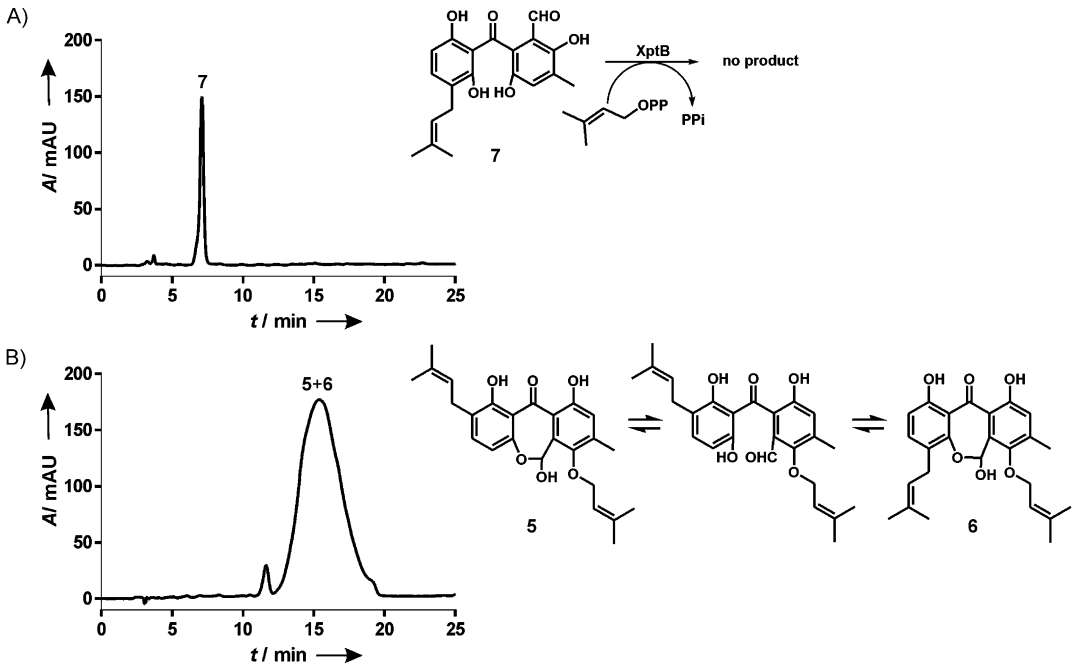


Figure 2. HPLC analysis of incubation mixtures of A) **7** with XptB, and B) standard **5**/**6**. Incubation conditions were as in Figure 1.

Table 2. Acceptance of benzophenones by XptB.

Benzophenone	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}	Conversion yield [%]
arugosin H (**7**)	CHO	OH	CH_3	H	OH	OH	C_5H_9O	H	H	OH	<0.03
maclurin (**24**)	OH	H	OH	H	OH	H	H	OH	OH	H	<0.05
1,5,8-trihydroxybenzophenone (**25**)	OH	H	H	H	OH	H	H	OH	H	H	<0.01
5-hydroxy-1,10-dimethoxy-6-carboxybenzophenon (**26**)	CH_3O	H	H	H	OH	COOH	H	H	H	CH_3O	<0.03
5-hydroxy-1,10-dimethoxy-6-carboxylmethylbenzophenone (**27**)	CH_3O	H	H	H	OH	$COOCH_3$	H	H	H	CH_3O	<0.03

adjacent to 7-OH, was poorly accepted by XptB (significantly higher K_m and lower k_{cat} than other accepted xanthones). When using the best-accepted xanthone (**21**) as the aromatic substrate, K_m 0.024 mM and k_{cat} 0.13 s^{-1} were determined for DMAPP (Table 4).

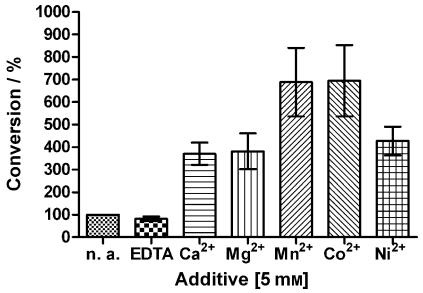


Figure 3. Ion dependency of the XptB reaction. To determine the ion dependency, 20 μg XptB-His_6 was incubated with 0.25 mM **14** and 1 mM DMAPP at 37 °C for 16 h. The activity with no additives (n.a.) was defined as 100%.

Discussion

In this study, we demonstrated that the putative prenyltransferase XptB from *A. nidulans* A4 catalyses, in the presence of DMAPP, regiospecific O-prenylation at C-7 of four methylated hydroxyxanthones, including its proposed substrate **14** (Table 1 and Figure 1). These results are in agreement with the proposed function of XptB by Sanchez and co-workers (pathway A in Scheme 1).[10] As mentioned above, a number of prenylated benzophenones, including **7**, have been isolated from different fungi such as *A. nidulans* and its teleomorph *E. nidulans*.[9,11,12] During the preparation of this manuscript, Hertweck and co-workers reported the isolation and identification of pre-shamixanthone, an O-prenylated arugosin H derivative with a benzophenone backbone from *A. nidulans* strain FGSC A4 (veA+).[24]

Table 3. ^{1}H NMR data of substrates and enzyme products of XptB in $CDCl_3$.

Position	Compound 14	1	19	28	21	29	22	30
		δ_H (ppm), multi., *J* [Hz]						
OH-1	12.80, s	12.66, s	12.69, s	12.80, s	13.09, s	13.09, s	13.13, s	13.13, s
2	6.75, dd, 8.4, 0.9	6.80, dd, 8.4, 0.9	6.77, dd, 8.2, 0.9	6.77, dd, 8.2, 0.9	6.74, dd, 8.2, 1.0	6.74, dd, 8.2, 1.0	6.72, dd, 8.2, 1.0	6.72, dd, 8.2, 1.0
3	7.59, t, 8.3	7.59, t, 8.3	7.56, t, 8.3	7.56, t, 8.3	7.51, t, 8.3	7.51, t, 8.3	7.52, t, 8.3	7.52, t, 8.3
4	6.86, dd, 8.4, 0.9	6.91, dd, 8.4, 0.9	6.91, dd, 8.4, 0.9	6.91, dd, 8.4, 0.9	6.82, dd, 8.4, 1.0	6.83, dd, 8.4, 1.0	6.88, dd, 8.4, 1.0	6.88, dd, 8.4, 1.0
5	7.25, s	7.31, s	7.28, s	7.28, s	7.13, s	7.14, s	–	–
8	–	–	7.52, s	7.53, s	–	–	–	–
11	2.40, s	2.46, s	2.42, s	2.38, s	2.40, s	2.44, s	2.36, s	2.40, s[a]
12	5.66, s	5.09, s	–	–	2.84, s	2.85, s	2.44, s	2.43, s[a]
13	–	–	–	–	–	–	2.82, s	2.84, s
1′	–	4.45, d, 7.2	–	4.64, d, 7.2	–	4.29, d, 7.2	–	4.25, d, 7.2
2′	–	5.61, t, 7.2	–	5.52, t, 7.2	–	5.60, t, 7.2	–	5.61, t, 7.2
4′	–	1.72, s	–	1.78, s	–	1.71, s	–	1.72, s
5′	–	1.81, s	–	1.81, s	–	1.79, s	–	1.82, s

[a] The assignment is interchangeable.

It was therefore expected that XptB would also accept **7** as a substrate and catalyse O-prenylation, as proposed by Simpson (pathway B in Scheme 1).[9] Compound **7** and four other benzophenones (**24**–**27**) were therefore assayed with XptB in the presence of DMAPP. No product formation was detected in the HPLC chromatogram of the incubation mixture with **7** (Figure 2), nor with the other tested benzophenones (Table 2). These results indicate the clear preference of XptB for methylated hydroxyxanthones. Therefore, the occurrence of prenylated benzophenones in *A. nidulans* cannot be explained by substrate promiscuity of XptB. One reason for these unexpected results is that orthologues of XptB from producers of prenylated benzophenones (e.g., some strains of *A. nidulans*) might have broader substrate specificity than that of the investigated enzyme from strain A4, and that they accept both xanthones and benzophenones as substrates. It can also be expected that only (prenylated) benzophenones are prenylation substrates for prenyltransferases in the biosynthetic pathway of the prenylated xanthones, as suggested by different research groups.[11,12] The resulting prenylated benzophenones will then be cyclised to prenylated xanthones.[11,12,24] It would be therefore interesting to investigate the substrate specificity of XptB orthologues from some producers of prenylated benzophenones. It was also suggested that different prenyltransferases are responsible for the formation of prenylated benzophenones and xanthones in a given strain.[25] Isolation of preshamixanthone from *A. nidulans* A4 strongly supports this hypothesis.[24] Seven putative genes encoding members of the DMATS superfamily were identified in the genome of *A. nidulans* A4.[10] Other than for XptB described in this study, biochemical investigation has only been carried out for TdiB, a prenyltransferase involved in the biosynthesis of terrequinone A.[26] Biochemical investigation of the other five putative prenyltransferases from *A. nidulans* would provide new insights into the relationship between prenylated benzophenones and xanthones.

XptB catalysed O-prenylation on xanthones and shares clear sequence similarity with members of the DMATS superfamily, which are mainly involved in the prenylation of diverse indole alkaloids.[18,19] Some of the previously identified enzymes from this superfamily also catalyse prenylations of other aromatic compounds, for example, tyrosine,[13] or a tetracyclic system derived from a polyketide.[27] Together with the results presented in this study, these findings demonstrate that members of the DMATS superfamily are involved not only in the biosynthesis of indole derivatives, which was speculated several years ago,[22] but also in the biosynthesis of a variety of different natural product groups.

One remarkable feature of the investigated enzyme XptB is its dependence on metal ions (Figure 3). Addition of 5 mM EDTA to the incubation mixture did not result in any inhibition of enzyme activity, consistent with almost all members of the DMATS superfamily.[18,22] It is known that divalent cations such as Ca^{2+} or Mg^{2+} can slightly en-

Table 4. Kinetic parameters of XptB for accepted substrates.

Substrate	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1}\,M^{-1}$]
1,7-dihydroxy-6-methyl-8-hydroxymethylxanthone (**14**)	0.327	0.43	1315
1,7-dihydroxy-6-methylxanthone (**19**)	1.147	0.02	17
1,7-dihydroxy-6,8-dimethylxanthone (**21**)	0.081	0.14	1728
1,7-dihydroxy-5,6,8-trimethylxanthone (**22**)	0.087	0.13	1494
DMAPP[a]	0.024	0.13	5417

[a] Kinetic parameters for DMAPP were measured with **21** as aromatic substrate.

hance the enzyme activity of DMATS prenyltransferases.[28] However, the drastic increase in enzyme activity (up to eightfold relative to that without additives) upon addition of 5 mM $MnCl_2$ or $CoCl_2$ has never been observed for a prenyl transfer reaction catalysed by an enzyme of the DMATS superfamily. Therefore, a clear difference in the active sites could be expected for XptB and other enzymes of this family.

Experimental Section

Computer-assisted sequence analysis: The software packages DNASIS (version 2.1; Hitachi Software Engineering, San Bruno, CA) and FGENESH (Softberry, Inc; http://www.softberry.com/berry.phtml) were used for intron prediction and sequence analysis, respectively. Alignments of amino acid sequences were carried out by using the program BLAST (http://blast.ncbi.nlm.nih.gov).

Chemicals: DMAPP was synthesised as trisammonium salt according to the method described for the preparation of trisammonium geranyl diphosphate.[29] Xanthones (**15**–**22**) were synthesised according to published protocols.[30,31] 1,7-Dihydroxy-6-methyl-8-formylxanthone (**23**) was prepared by formylation of 1,7-dihydroxy-6-methylxanthone (**19**) with hexamethylentetramine (HMTA) in acetic acid.[32] Reduction of **23** with $NaBH_4$ in methanol/CH_2Cl_2 (1:1) at 0 °C yielded 1,7-dihydroxy-6-methyl-8-hydroxymethylxanthone (**14**). Arugosins A/B (**5**/**6**) and H (**7**) were isolated from *E. nidulans*.[11] Maclurin (**24**) was obtained from ABCR (Karlsruhe, Germany); **25** was prepared from hydroquinone and 2,6-dihydroxybenzoic acid;[31] benzophenones **26** and **27** were synthesised according to methods described previously.[33,34] Structures of the synthesised compounds were confirmed by NMR and mass analyses.

Bacterial strains, plasmids and culture conditions: The expression vector pQE60 was obtained from Qiagen (Hilden, Germany). *E. coli* strain XL1 Blue MRF′ (Stratagene, Amsterdam, the Netherlands) was used for cloning and gene expression. Bacteria harbouring the vectors were grown in liquid or on solid lysogeny broth (LB) supplemented with agarose (1.5% *w*/*v*) at 37 °C. Carbenicillin (50 μg mL^{-1}) was used for selection of *E. coli* recombinant transformants.

DNA isolation and cloning: Standard procedures for DNA isolation and manipulation in *E. coli* were performed as described elsewhere.[35] Genomic DNA for PCR amplification was isolated from *A. nidulans* strain FGSC A4 by using phenol/chloroform extraction. The entire coding sequence of *xptB* was obtained after two consecutive rounds of PCR with genomic DNA from *A. nidulans* as template. PCR amplification was carried out on an iCycler thermal cycler (BioRad, Hercules, CA). In the first PCR round, the two exons were amplified separately with the following primer pairs: XptB_NcoI (5′-CG**CCAT GG**TTTT GCACAA ACGGTC CTTG-3′) and XptB_2 (5′-GCGGGG CCTAGA TAAGGC GCAGCG TAGTGG TAATGG TACA-3′) for the 5′ exon, and XptB_3 (5′-TGTACC ATTACC ACTACG CTGCGC CTTATC TAGGCC CCGC-3′) and XptB_BamHI (5′-GAG**GGA TCC**CCA CCGATC ATCCCC CCTCC-3′) for the 3′ exon (bold letters represent introduced restriction sites for subsequent cloning, underlined letters identify overlapping regions for fusion of PCR products). The amplified PCR products were used in an equimolar ratio for a second round PCR with XptB_NcoI and XptB_BamHI as primers. The resulting PCR product was cloned into pGEM-T Easy vector (Promega) to give plasmid pDP007, and the latter was subsequently sequenced to confirm sequence integrity. In order to create an expression vector for *xptB*, the coding sequence was excised from pDP007 by digestion with NcoI and BamHI. The NcoI–BamHI fragment (1376 bp) was then isolated from agarose gel and ligated into pQE60 that had previously been digested with NcoI and BamHI, thereby yielding expression plasmid pDP008.

Overexpression and purification of XptB-His$_6$: For gene expression, *E. coli* XL1 Blue MRF′ cells harbouring plasmid pDP008 were cultivated in 2000 mL Erlenmeyer flasks containing liquid LB (1000 mL) supplemented with carbenicillin (50 μg mL^{-1}) at 37 °C and 220 rpm, to $A_{600} = 0.6$. For gene induction, IPTG was added to a final concentration of 1.0 mM. The bacterial culture was cultivated for a further 16 h and centrifuged to harvest the cells, which were then resuspended in lysis buffer (imidazole (10 mM), NaCl (300 mM), NaH_2PO_4/Na_2HPO_4 (50 mM, pH 8.0)) at 1.4 mL per gram of pellet. After addition of lysozyme (1 mg mL^{-1}) and incubation on ice for 30 min, the cells were sonicated (6× 10 s, 200 W). To separate cellular debris from soluble proteins, the lysate was centrifuged (14000 *g*, 30 min, 4 °C). One-step purification of the recombinant XptB-His$_6$ by affinity chromatography with Ni-NTA agarose resin (Qiagen) was carried out according to the manufacturer's instruction. Proteins was eluted with elution buffer (imidazole (250 mM), NaCl (300 mM), NaH_2PO_4/Na_2HPO_4 (50 mM, pH 8.0)). For buffer exchange, the protein fractions were passed through PD-10 columns (GE Healthcare, Freiburg, Germany) that had been equilibrated with Tris·HCl (50 mM, pH 7.5) and glycerol (15% *v*/*v*). The proteins were eluted with the same buffer, frozen in liquid nitrogen and stored at −80 °C until use. The protein fractions obtained from the purification process were analysed on SDS-PAGE according to the method of Laemmli[36] and stained with Coomassie Brilliant Blue R-250.

Enzyme assays: To determine the relative activity, enzyme assays (200 μL) contained Tris·HCl buffer (50 mM, pH 7.5), aromatic substrate (0.25 or 0.5 mM), DMAPP (1 mM), XptB (20 μg), $MnCl_2$ (5 mM), glycerol (0.8% *v*/*v*) and dimethyl sulfoxide (DMSO; 2% *v*/*v*). The reaction mixtures were incubated at 37 °C with mild agitation on a shaker for 16 h unless otherwise stated. After incubation, enzyme assays were extracted twice with two volumes of ethyl acetate, and the combined organic phases were evaporated to dryness in a vacuum centrifuge. The residue was dissolved in methanol (150 μL) and analysed by HPLC. For structure elucidation, enzyme assays were scaled up (20 mL), and the reaction mixtures contained Tris·HCl buffer (50 mM, pH 7.5), xanthone (500 μM), DMAPP (1 mM), $MnCl_2$ (5 mM) and XptB (2 mg), and were incubated at 37 °C for 16 h. The assays for determination of the kinetic parameters of xanthones used DMAPP (2 mM), XptB (20 μg), $MnCl_2$ (5 mM) and xanthone (0.025–2.0 mM). Assays were incubated at 37 °C for 120 min. For determination of the kinetic parameters of DMAPP, the following reagents were used: XptB (20 μg), 1,7-dihydroxy-6,8-

dimethylxanthone (**21,** 0.5 mM), $MnCl_2$ (5 mM) and DMAPP (0.01–0.4 mM). Incubation was carried out at 37 °C for 120 min.

HPLC analysis and isolation of enzyme products: Reaction mixtures were analysed on a Series 1200 HPLC equipment (Agilent, Foster City, CA) with a Multospher 120 RP-18 column (250×4 mm, 5 μm; CS Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 mL min^{-1} with water (solvent A) and methanol (solvent B). The reaction mixtures were separated with a gradient of 65–100% B for 15 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 65% B for 5 min. The substances were detected with a photo diode array detector. For isolation of the enzyme products, the same HPLC equipment with a semipreparative Multospher 120 RP-18 column (250×10 mm, 5 μm) was used at a flow rate of 2.5 mL min^{-1}.

The conversion yield was calculated from the decrease in peak area of the substrate in the HPLC chromatogram. A linear correlation was found for the peak areas measured at 254 or 260 nm with 550 nm reference and substrate standard concentrations between 0.025–2 mM.

NMR and mass spectroetric analysis: Isolated enzyme products were dissolved in $CDCl_3$ (0.7 mL). Spectra were recorded at room temperature on a ECX-500 spectrometer (JEOL, Tokyo. Japan) equipped with a broadband probe with z-gradient. Chemical shifts were referenced to the $CDCl_3$ signal at 7.26 ppm. All spectra were processed with MestReNova 5.2.2 (Metrelab Research, Santiago de Compostela, Spain). NMR data for the substrates and isolated products are given in Table 3.

The isolated enzyme products were also analysed by high-resolution electrospray ionization (HR-ESI) MS with a Q-Trap Quantum (Applied BioSystems) or by high-resolution electron impact (HR-EI) MS on a Micromass Auto Spec spectrometer (Waters, Milford, MA). MS data are given in Table S1.

Acknowledgements

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Keywords: *Aspergillus nidulans* · DMATS superfamily · enzymes · natural products · prenylated xanthones · prenyltransferases

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Supporting Information

New Insights into the Biosynthesis of Prenylated Xanthones: Xptb from *Aspergillus nidulans* Catalyses an O-Prenylation of Xanthones

Daniel Pockrandt,[a] Lena Ludwig,[a] Aili Fan,[a] Gabriele M. König,[b] and Shu-Ming Li*[a]

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Table S1: MS data of the enzyme products of XptB

Compound	Chemical formula	HR-MS data			Deviation (ppm)
		MS mode	Calculated	Measured	
1	$C_{20}H_{20}O_5$	HR-ESI-MS	363.1208 $[M+Na]^+$	363.1201	1.9
28	$C_{19}H_{18}O_4$	HR-EI-MS	310.1205	310.1204	0.3
29	$C_{20}H_{20}O_4$	HR-EI-MS	324.1361	324.1364	−0.9
30	$C_{21}H_{22}O_4$	HR-EI-MS	338.1518	338.1520	−0.6

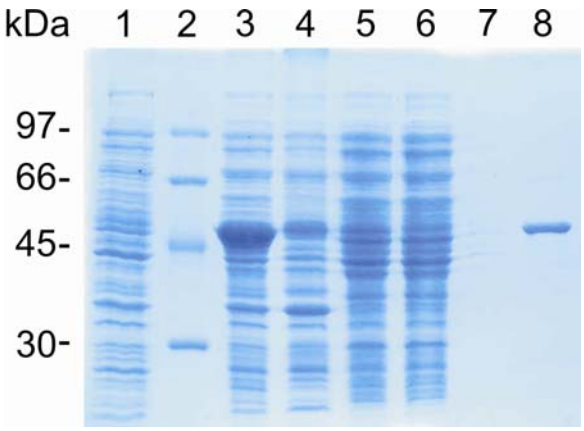

Figure S1: SDS-PAGE analysis of the overproduction and purification of XptB-His_6

The proteins were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. Lane 1: total protein before induction, Lane 2: molecular mass standard, Lane 3: total protein after induction, Lane 4: insoluble protein fraction, Lane 5: soluble protein fraction, Lane 6: flow-through, Lane 7: wash fraction and Lane 8: purified XptB-His_6.

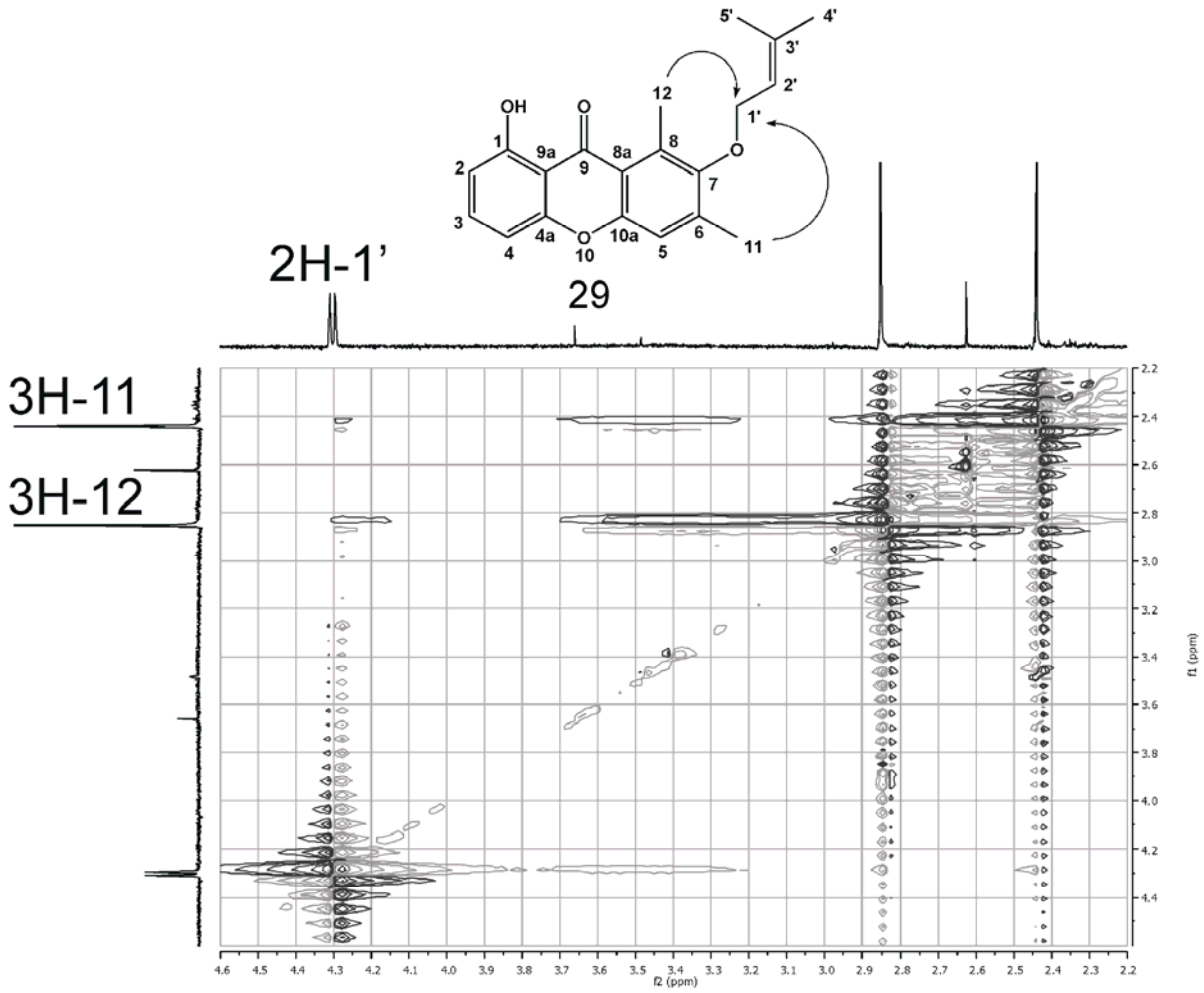

Figure S2: NOESY correlation of **29**

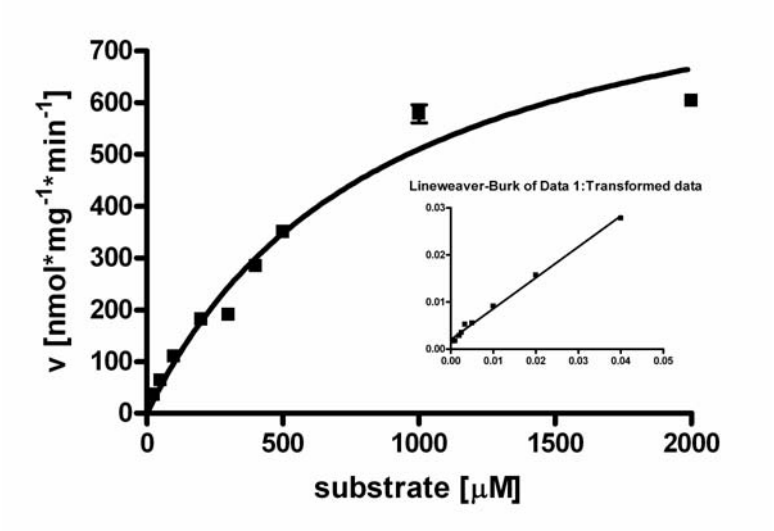

Figure S3: Determination of kinetic parameters for **14**

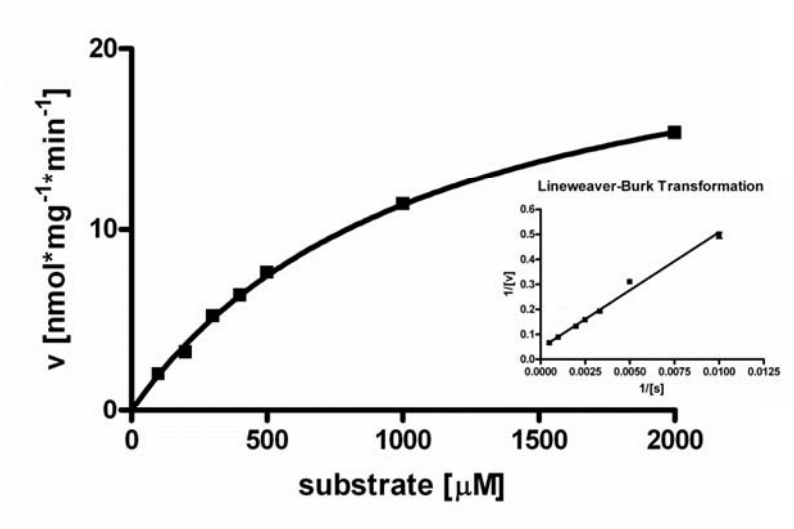

Figure S4: Determination of kinetic parameters for **19**

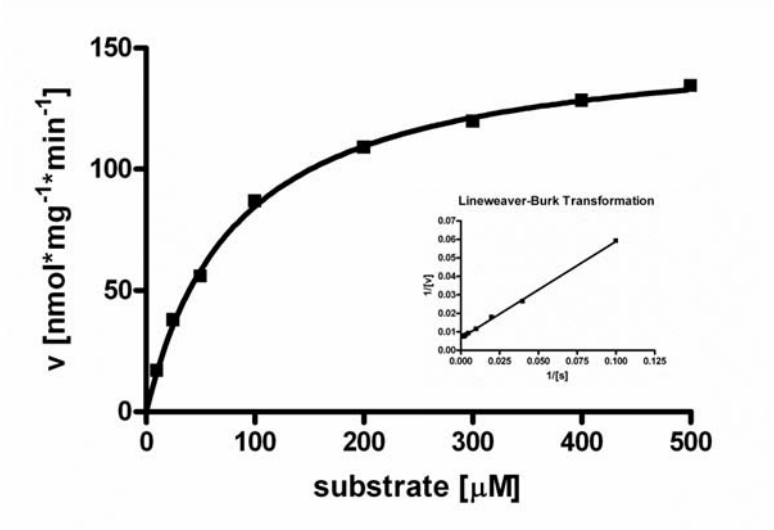

Figure S5: Determination of kinetic parameters for **21**

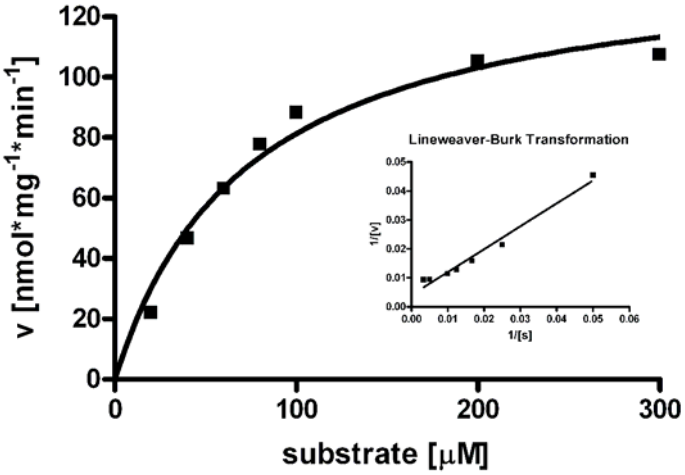

Figure S6: Determination of kinetic parameters for **22**

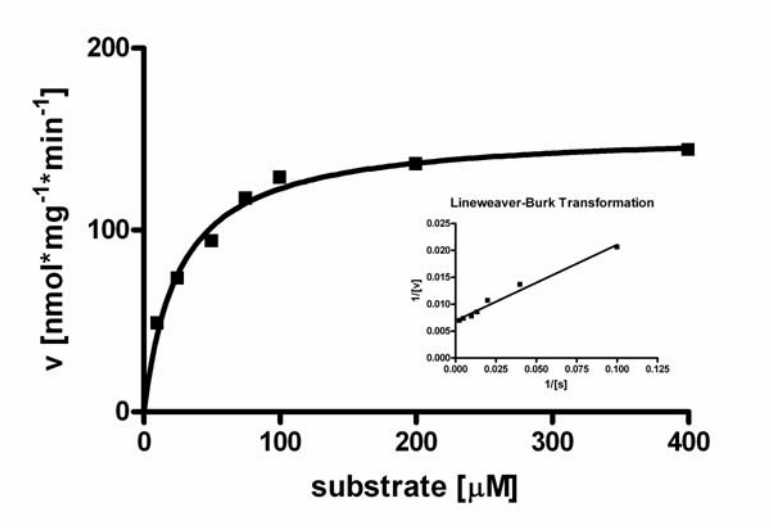

Figure S7: Determination of kinetic parameters for DMAPP. Incubation was carried out with **21** as aromatic substrate.

4.2 Geranylation of cyclic dipeptides by the dimethylallyl transferase AnaPT resulting in a shift of prenylation position on the indole ring

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Geranylation of Cyclic Dipeptides by the Dimethylallyl Transferase AnaPT Resulting in a Shift of Prenylation Position on the Indole Ring

Daniel Pockrandt and Shu-Ming Li*[a]

The dimethylallyl transferase AnaPT from *Neosartorya fischeri* is involved in the biosynthesis of acetylaszonalenin and catalyses the regioselective and stereospecific C3α-prenylation of (*R*)-benzodiazepinedione in the presence of dimethylallyl diphosphate. This enzyme also converts several tryptophan-containing cyclic dipeptides to C3α-prenylated indolines. In this study, we demonstrate the geranylation of (*R*)-benzodiazepinedione and five other cyclic dipeptides by AnaPT in the presence of geranyl diphosphate (GPP). Interestingly, structure elucidation by NMR and MS analyses revealed that, with GPP, the geranyl moiety is attached to C-6 or C-7 rather than C-3 of the indole ring of the enzyme products. For (*R*)-benzodiazepinedione, one dominant C6-geranylated derivative was obtained, whereas the other five substrates yielded both C6- and C7-geranylated products. Neither acceptance of GPP by a dimethylallyl transferase from the dimethylallyltryptophan synthase superfamily, nor the alkylation shift from C-3 to the benzene ring of the indole nucleus has been reported previously.

Introduction

Attachment of a prenyl moiety to aromatic compounds contributes significantly to the structural diversity of secondary metabolites, including prenylated indole alkaloids.[1] Natural products of this group are widely distributed in both terrestrial and marine microorganisms.[2–4] In most cases, they are derivatives of tryptophan or tryptophan-containing cyclic dipeptides with one or more dimethylallyl moieties.[2,3] However, indole derivatives with larger prenyl moieties have also been found in nature, for example, indole sesquiterpenoid xiamycin and derivatives from *Streptomyces*,[5,6] and indole diterpenoids paxilline and aflatrem from *Penicillium* and *Aspergillus* species.[7–9] In many cases, the prenylated derivatives differ clearly from their precursors in their biological and pharmacological activities.[10–12] Prenyltransferases from plants, bacteria and fungi are responsible for the transfer reactions of the prenyl moieties, and belong to different superfamilies/groups, based on their primary amino acid sequences and biochemical properties.[1,13,14] Indole prenyltransferases of the DMATS superfamily show broad substrate specificity towards their aromatic substrates but high regioselectivity for the prenylation position on the indole ring.[14] These characteristics render members of this superfamily a valuable toolbox for the production of prenylated indole derivatives. In a sharp contrast to the flexibility for aromatic substrates, most of the enzymes accept exclusively dimethylallyl diphosphate (DMAPP, C_5 unit) as the prenyl donor.[14] Only one enzyme from the DMATS superfamily, VrtC from *Penicillium aethiopicum*, is known to use geranyl diphosphate (GPP, C_{10} unit), rather than DMAPP, as the prenyl donor.[15] Interestingly, several homologues of VrtC from *Neosartorya fischeri*, *Microsporum canis, Aspergillus fumigatus* and *Trichophyton tonsurans* catalyse similar prenylation reactions, but these use exclusively DMAPP as the prenyl donor.[15,16] This clearly demonstrates the high specificity of these enzymes for their prenyl donors. Yet, it can be speculated that the prenyl diphosphate binding sites of the different members in this superfamily are derived from the same ancestor, or evolved by successive modifications. In both cases, enzymes that accept both DMAPP and GPP should exist within the DMATS superfamily. Therefore, we tested the acceptance of GPP and farnesyl diphosphate (FPP) by several known prenyltransferases from different fungi,[14] for example, 7-DMATS, CdpC3PT, CdpNPT and AnaPT, in the presence of their natural (or best accepted) aromatic substrates, that is, tryptophan or tryptophan-containing cyclic dipeptides. HPLC analysis revealed that additional peaks were only detected in the incubation mixtures of AnaPT with GPP. AnaPT was identified in the genome sequence of *N. fischeri* NRRL181 by genome mining and catalyses the C3α reverse prenylation of (*R*)-benzodiazepinedione (**1a**) in the presence of DMAPP (Scheme 1). It was then shown that AnaPT also accepts **2a** (the enantiomer of **1a**) and tryptophan-containing cyclic dipeptides with a diketopiperazine skeleton, for example, **3a**–**6a** (Figure 1). The reactions are C3α reverse prenylations in all these cases.[17,18] Here, we report the acceptance of GPP by AnaPT and identification of C6- and C7-prenylated derivatives as the enzyme products.

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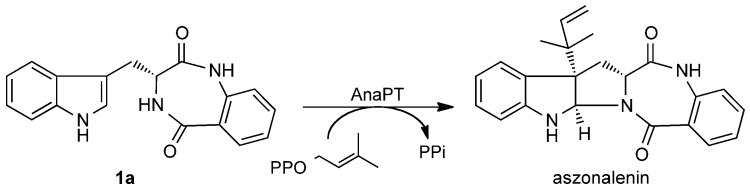


Scheme 1. C3α prenylation of **1a** by AnaPT in the presence of DMAPP.

Results

Acceptance of GPP by AnaPT

AnaPT was assayed with six tryptophan-containing cyclic dipeptides (**1a**–**6a**), including its natural substrate (*R*)-benzodiazepinedione (**1a**) in the presence of GPP or FPP. Enzymatic conversion was observed in all of the six incubation mixtures with GPP (Figure 1). Product formation was strictly dependent on the presence of active enzyme and GPP. Negative controls with heat-inactivated enzyme (boiled for 30 min) showed only substrate peaks in the HPLC chromatograms (data not shown). No conversion was detected for enzyme assays with FPP as prenyl donor (data not shown). Initial HPLC analysis of the GPP reaction mixtures of **2a**–**6a** with a reversed-phase C-18 column revealed only a single product peak for each; this was proven to be mixtures of at least two compounds after purification and NMR analysis (data not shown). Analysis of these reaction mixtures by HPLC with a normal-phase column clearly showed the presence of two or more enzyme products (Figure 1). Therefore, we used a normal-phase HPLC column for analysis and purification of the enzyme products. As shown in Figure 1, **1a** and its enantiomer **2a** were very well accepted by AnaPT in the presence of GPP. In the incubation mixture of **1a**, one dominant enzyme product (**1b**) was detected after incubation for 16 h, with a conversion yield of 88%. The reaction mixture of **2a** showed two dominant product peaks, **2b** and **2c** (ratio 1:2), with a total conversion yield of 91% (slightly higher than that for **1a**). Two major peaks were also detected in the reaction mixtures of cyclo-L-Trp-L-Leu (**3a**), cyclo-L-Trp-L-Trp (**4a**), cyclo-L-Trp-L-Tyr (**5a**) and cyclo-D-Trp-L-Tyr (**6a**). The total conversion yields were 33–72%. In the incubation mixtures of L-tryptophan-containing substrates (**2a**–**5a**), the yield of the b-series product (with a larger retention time) was clearly lower than that of the c-series product (shorter retention time). In contrast, **6a** (D-tryptophanyl moiety) produced more b-series product than c-series. In addition to **1b** (the dominant product), another product, **1c**, with an identical retention time to that of **2c** was detected at 14.2 min in the incubation mixture of **1a**.

AnaPT catalyses both C6- and C7-geranylation of tryptophan-containing cyclic dipeptides

For structure elucidation, large scale incubations with **1a**–**6a** were carried out in the presence of GPP. In total, six b-series (**1b**–**6b**) and five c-series products (**2c**–**6c**) were isolated and subjected to MS and NMR analyses. HR-EI-MS data (Table 1) showed molecular ions that were 136 kDa larger than those of the respective substrates, thus indicating the presence of a geranyl moiety in the enzyme products. Inspection of the ^{1}H NMR spectra (Figures S1–S11 in the Supporting Information) revealed indeed the presence of signals for a geranyl moiety (Tables S1 and S2). The signals of H-1′ of the geranyl moiety in the range 3.3–3.6 ppm proved its attachment to the indole ring through a C–C bond. The signals of H-2 of the indole ring (range 6.5–7.2 ppm as a singlet or a doublet with a small coupling constant of approximate 2 Hz) were found in all of the spectra, thus proving geranylation not at C-2 or C-3, but on the benzene ring of the indole unit. Comparison of the spectra revealed, furthermore, that the signals of the geranylated indole ring in the b-series products (**1b**–**6b**) had a same order and similar coupling patterns. This phenomenon was also observed for the c-series products (**2c**–**6c**). The order and coupling patterns of the signals for the aromatic protons in **1b**–**6b** clearly differ from those of **2c**–**6c**. The signals of the three protons on the geranylated benzene ring in **1b**–**6b** appeared as two doublets with a coupling constant of approximate 8 Hz and one (broad) singlet (Table 2). In some cases, the doublet in the high magnetic field was further coupled with a proton (coupling constant 1.3 Hz). This coupling pattern indicated geranylation at C-5 or C-6. The order of the signals for the four protons of the indole ring of **1b**–**6b** (that is, the two singlets between the two doublets) corresponds very well to that of the known C6-, but clearly differs from that of C5-prenylated or alkylated indole derivatives.[19–21] This unequivocally proves C6-geranylation of the b-series products (**1b**–**6b**).

The signals of the three coupling protons on the geranylated benzene ring in **2c**–**6c** appeared, from low to high magnetic field, as doublet, triplet and doublet, with coupling constants of approximate 7–8 Hz (Table 3). In the case of **2c**,

Table 1. HR-EI-MS data of the isolated enzyme products.

Compound	Chemical formula	HR-MS data Calcd $[M]^+$	Measured	Deviation (ppm)
1b	$C_{28}H_{31}N_3O_2$	441.2416	441.2396	4.6
2b	$C_{28}H_{31}N_3O_2$	441.2416	441.2434	−4.1
2c	$C_{28}H_{31}N_3O_2$	441.2416	441.2423	−1.6
3b	$C_{27}H_{37}N_3O_2$	435.2885	435.2894	−2.0
3c	$C_{27}H_{37}N_3O_2$	435.2885	435.2972	−4.9
4b	$C_{32}H_{36}N_4O_2$	508.2838	508.2851	−2.6
4c	$C_{32}H_{36}N_4O_2$	508.2838	508.2844	−1.1
5b	$C_{30}H_{35}N_3O_3$	485.2678	485.2644	6.9
5c	$C_{30}H_{35}N_3O_3$	485.2678	485.2676	0.4
6b	$C_{30}H_{35}N_3O_3$	485.2678	485.2689	−2.3
6c	$C_{30}H_{35}N_3O_3$	485.2678	485.2695	−3.6

Figure 1. HPLC analysis of enzyme assays with AnaPT, GPP and cyclic dipeptides, and conversion yields of the enzyme products. Standard incubation mixtures contained 20 μg AnaPT-His_6, 1 mM aromatic substrate, 1 mM GPP, 5 mM $CaCl_2$, 50 mM Tris·HCl pH 7.5 and were incubated at 37 °C for 16 h. The illustrated chromatograms were recorded at 277 nm. Retention times are given for substrate and products.

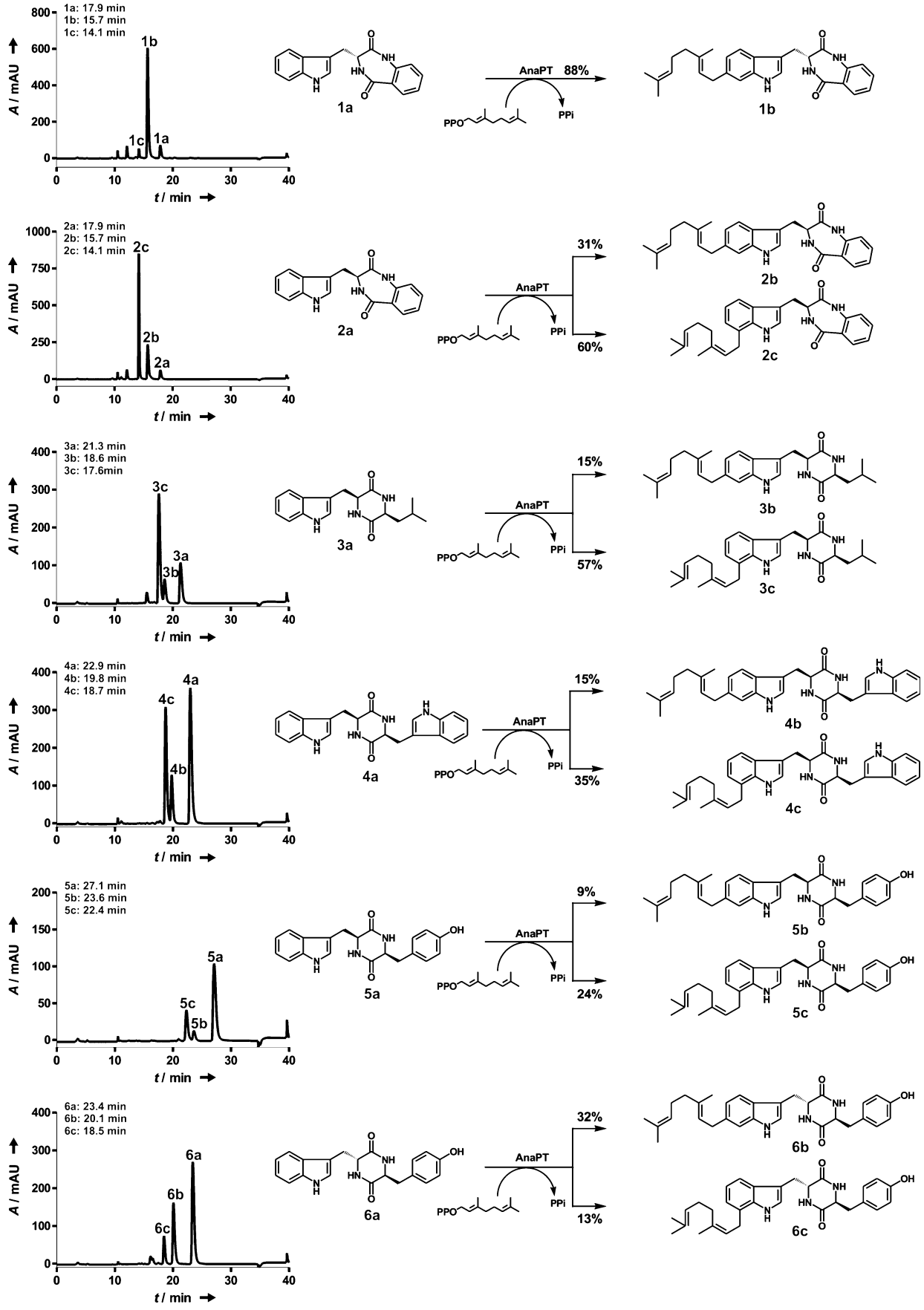
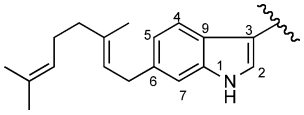
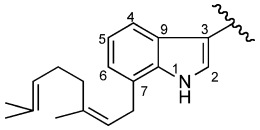

1a: 17.9 min
1b: 15.7 min
1c: 14.1 min
A / mAU
t / min
1a
1b
1c
AnaPT
88%
PPO
PPi
2a: 17.9 min
2b: 15.7 min
2c: 14.1 min
2a
2b
2c
31%
60%
3a: 21.3 min
3b: 18.6 min
3c: 17.6min
3a
3b
3c
15%
57%
4a: 22.9 min
4b: 19.8 min
4c: 18.7 min
4a
4b
4c
35%
5a: 27.1 min
5b: 23.6 min
5c: 22.4 min
5a
5b
5c
9%
24%
6a: 23.4 min
6b: 20.1 min
6c: 18.5 min
6a
6b
6c
32%
13%

Table 2. Chemical shifts (ppm) of protons on the indole ring of the C6-geranylated derivatives (b-series).



Compound	H-4	H-7	H-2	H-5
1b	7.41, d, 8.1	7.16, brs	7.11, d, 2.3	6.79, d, 8.1
2b	7.41, d, 8.1	7.17, brs	7.11, d, 2.3	6.94, dd, 8.1, 1.3
3b	7.52, d, 8.1	7.17, brs	7.04, d, 2.3	6.99, dd, 8.1, 1.3
4b	7.56, d, 8.2	7.14, s	6.51, s	6.99, d, 8.2
5b	7.34, d, 8.1	7.05, s	6.87, d, 2.0	6.78, d, 8.1
6b	7.38, d, 8.1	7.03, s	6.89, d, 2.3	6.73, dd, 8.1, 1.3

Table 3. Chemical shifts of protons on the indole ring of the C7-geranylated derivatives (c-series).



Compound	H-2	H-4	H-5	H-6
2c	7.16, d, 2.3	7.38, dd, 7.5, 0.8	7.04, t, 7.5	7.01, dd, 7.1, 1.2
3c	7.07, s	7.49, d, 7.7	7.07, t, 7.7	7.03, d, 7.1
4c	6.53, d, 2.2	7.41, d, 8.2	7.07, t, 7.2	7.03, d, 7.1
5c	6.94, d, 2.1	7.27, d, 7.7	6.87, t, 7.7	6.81, d, 7.0
6c	6.96, d, 2.3	7.32, d, 7.8	6.83, t, 7.8	6.77, d, 7.2

a long-range coupling was also observed. This indicates C4- or C7-geranylation. Comparison of the chemical shifts of the three coupling aromatic protons in **2c**–**6c** with those of C4- and C7-prenylated tryptophan-containing cyclic dipeptides[22,23] revealed the disappearance of the signals for H-7 in **2c**–**6c**, which were therefore identified as C7-geranlyated derivatives.

Determination of the kinetic parameters for AnaPT in the presence of GPP

Kinetic parameters including Michaelis–Menten constant (K_m) and turnover number (k_{cat}) were determined for GPP as well as for **1a**–**6a** (Table 4, Figure S12–S18). The AnaPT reactions apparently followed Michaelis–Menten kinetics, including when in the presence of GPP. In the presence of its natural aromatic substrate, (*R*)-benzodiazepinedione (**1a**), a K_m value of 0.16 mM was calculated, identical to that for DMAPP with GPP.[24] With GPP as the prenyl donor, a K_m value of 0.25 mM was determined for **1a**, very similar to that with DMAPP (0.23 mM).[24] However, the turnover number (0.05 s^{-1} with GPP and **1a)** was significantly lower than that with DMAPP (1.5 s^{-1}). Higher K_m values (0.35–2 mM) were determined for the other aromatic substrates. It is evident that tyrosine-containing substrates **5a** and **6a** were converted with lower reactivity than the other substrates (Figure 1 and Table 4).

Table 4. Kinetic parameters of AnaPT in the presence of GPP.

Subs.	K_m [mM]		k_{cat}[s^{-1}]		k_{cat}/K_m [s^{-1} M^{-1}]	
	b-series	c-series	b-series	c-series	b-series	c-series
1a	0.25 ± 0.03	–	0.05 ± 0.002	–	199	–
2a	0.80 ± 0.09	0.65 ± 0.04	0.10 ± 0.0008	0.17 ± 0.004	126	262
3a	2.11 ± 0.23	1.78 ± 0.17	0.07 ± 0.008	0.29 ± 0.01	33	162
4a	0.76 ± 0.06	0.83 ± 0.05	0.09 ± 0.006	0.22 ± 0.002	119	266
5a	0.38 ± 0.03	0.47 ± 0.04	0.002 ± 0.0002	0.007 ± 0.0004	5	15
6a	0.39 ± 0.02	0.35 ± 0.04	0.01 ± 0.0008	0.004 ± 0.0002	26	11
GPP[a]	0.16 ± 0.03	–	0.05 ± 0.001	–	307	–

[a] Kinetic parameters for GPP were measured with **1a** as aromatic substrate.

Discussion

As mentioned, AnaPT belongs to prenyltransferases of the DMATS superfamily and is involved in the biosynthesis of acetylaszonalenin in *N. fischeri*. This enzyme catalyses the C3α-prenylation of (*R*)-benzodiazepinedione (**1a**), thereby resulting in formation of aszonalenin.[24] C3α-prenylation was also observed with a number of tryptophan-containing cyclic dipeptides, including **2a**–**6a,** in the presence of DMAPP.[18,25] In general, members of the DMATS superfamily use DMAPP, but not other prenyl diphosphates (e.g. GPP or FPP), as prenyl donor.[14] We have recently demonstrated that the DMAPP structure can be modified by a shift or deletion of a methyl group, but the double bond at the β-position to pyrophosphate seems to be essential as alkyl donor.[19,26] Acceptance of such unnatural alkyl donors by several prenyltransferases from this family proves that the binding sites for DMAPP in these enzymes are flexible up to a certain degree.[19,26] Identification of the geranyl transferase VrtC from *P. aethiopicum*[15] indicates that the prenyl donors of these enzymes are not limited to DMAPP in nature. Acceptance of GPP by the dimethylallyl transferase AnaPT described in this study provides experimental evidence for the relationship of DMAPP- and GPP-accepting enzymes regarding the binding sites in the evolution of this family. It is also possible that some members of the DMATS superfamily are capable of utilising DMAPP, GPP and FPP as prenyl donors; this should be investigated in the near future.

Identification of the enzyme products of the six selected substrates as C6- and C7-geranylated is somewhat surprising for the C3-prenyltransferase AnaPT. However, shifts of the prenylation position and reduction of regioselectivity of prenyltransferases have also been observed with unnatural prenyl donors.[19,26] Comparison of the kinetic parameters for **1a** in the presence of GPP obtained in this study with those in the presence of DMAPP revealed similar affinity to the enzyme, thus indicating no significant change in the binding site for the aromatic substrate. Interestingly, GPP has an affinity similar to that of DMAPP. However, the AnaPT reaction was approximately 30 times faster with its natural substrate (DMAPP) than with GPP. The difference be-

tween the prenylation position with GPP (this study) to that with DMAPP (previously reported) might be explained as follows. In case of DMAPP, its C-1′ is already at such a distance to C-3 of the indole moiety that the nucleophilic attack of this C-atom has to take place at C-3′ of the dimethylallyl cation after the cleavage of pyrophosphate from DMAPP. If, now, the large geranyl moiety binds in the same orientation as DMAPP, the distance of the C-1′ of GPP to C-3 of indole is even larger, and the only candidate for a nucleophilic attack seems to be the benzene ring.

Another interesting phenomenon observed in this study is the dependence of the ratios of C6- to C7-geranylated derivatives on the stereochemistry of the tryptophanyl moiety of the substrates. As mentioned above, C7-geranylated derivatives (c-series) were found as the main products in the incubation mixtures of L-tryptophan-containing substrates **2a**–**5a**, and C6-geranylated (b-series) were the main products for D-tryptophan-containing **6a** (Figure 1). This observation was confirmed by determination of the turnover number of the b- and c-series of the respective substrate (Table 4, Figures S12–S18). In the case of the D-tryptophan-containing **1a**, **1b** was detected as the predominant product. One minor peak (**1c**) with a same retention time as **2c** might indicate the presence of a C7-geranylated substance (which would be the enantiomer of **2c**) in the enzyme assay of **1a**. Because of the low quantity, this peak was not isolated and characterised. The observation of different ratios for C6- and C7-geranylated products indicates a slightly different placement of the aromatic substrates and therefore changed distance of C-6 and C-7 to the geranyl moiety. The number of the investigated substrates is of course too small to conclude the structural feature as main reason for the observed ratio of the b- and c-series products.

Conclusions

In this study, we demonstrated acceptance of GPP as substrate by a dimethylallyl transferase. To the best of our knowledge, acceptance of both DMAPP and GPP by an enzyme of the dimethylallyltryptophan synthase superfamily has not been reported before. In addition, the previously observed regioselectivity of the investigated enzyme was reduced by changing the prenyl donor, and two main products were detected in five of the six enzyme reactions. Furthermore, with GPP the prenylation position by AnaPT shifted from C-3 with DMAPP to C-6 or C-7 on the indole ring.

Experimental Section

Chemicals: Geranyl diphosphate was synthesised according to the method described by Woodside et al.[27] (*R*)-benzodiazepinedione (**1a**) and (*S*)-benzodiazepinedione (**2a**) were synthesised by condensation of D- or L-tryptophan with isatoic anhydride in the presence of triethylamine according to the method of Barrow and Sun.[28] Other cyclic dipeptides (**3a**–**6a**) were obtained from Bachem (Bubendorf, Switzerland).

Overexpression and purification of AnaPT-His$_6$ as well as enzyme assays: AnaPT-His$_6$ was overexpressed and purified as described previously.[24] In order to test the acceptance of GPP by AnaPT, enzyme assays (100 μL) containing aromatic substrate (1 mM), GPP (1 mM), $CaCl_2$ (5 mM), glycerol (2%, *v/v*), AnaPT-His$_6$ (20 μg) and Tris·HCl (50 mM, pH 7.5) were incubated at 37 °C for 16 h with mild agitation on a shaker. After incubation, the assay mixture was extracted with ethyl acetate (2×200 μL), and the combined organic phases were evaporated to dryness in a vacuum centrifuge. The residues were dissolved in $CHCl_3$ (150 μL) and analysed by HPLC as described below. For structure elucidation, enzyme assays were scaled up to 40 mL and the reaction mixtures contained aromatic substrate (1 mM), GPP (2 mM), $CaCl_2$ (5 mM), glycerol (2%, *v/v*), AnaPT-His$_6$ (6 mg) and Tris·HCl (50 mM, pH 7.5), and were incubated at 37 °C for 16 h. For determination of kinetic parameters of aromatic substrates, enzyme assays contained AnaPT-His$_6$ (20 μg), $CaCl_2$ (5 mM), GPP (1 mM), glycerol (2%, *v/v*) and aromatic substrate (0.025–4 mM), and were incubated at 37 °C for 120 min with mild agitation. For determination of the GPP dependency, the same dilution steps were used for GPP as for the aromatic substrates, with a fixed concentration of **1a** (1 mM). Conversion yields (%) were calculated from peak areas of products and substrate in HPLC chromatograms.

HPLC analysis: Reaction mixtures were analysed in an Agilent Series 1200 HPLC (Agilent, Foster City, CA) with a MultoHigh 100 Si column (250×4 mm, 5 μm; CS Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 mL min^{-1} with chloroform (solvent A) and methanol (solvent B). The reaction mixtures were separated with a linear gradient of 0–10% B for 30 min. After washing with 50% B for 5 min, the column was equilibrated with 100% A for 5 min. The substances were detected with a photodiode array detector and illustrated for absorption at 277 nm. The same HPLC equipment and solvent gradient were used for isolation of the enzyme products.

NMR and mass spectrometric analyses: For structural elucidation, the isolated enzyme products were subjected to ^{1}H NMR spectroscopic and MS analyses. For NMR analysis, the enzyme products were dissolved in $CDCl_3$ (150 μL; **1b**–**4b** and **2c**–**4c**) or [D_6]DMSO (150 μL; **5b**, **5c**, **6b** and **6c**). Spectra were recorded at room temperature with an ECX-500 spectrometer (JEOL, Tokyo, Japan) equipped with a broadband probe with z-gradient. Chemical shifts were referenced to $CHCl_3$ (7.26 ppm) or DMSO (2.50 ppm). All spectra were processed with MestReNova 5.2.2 (Metrelab Research, Santiago de Compostella, Spain). NMR data for the isolated products are given in Tables S1 and S2. The enzyme products were also analysed by HR-EI-MS. MS data are given in Table 1.

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Keywords: cyclic dipeptides · enzyme catalysis · geranylation · prenyltransferases · reaction mechanisms

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Supporting Information

Geranylation of Cyclic Dipeptides by the Dimethylallyl Transferase AnaPT Resulting in a Shift of Prenylation Position on the Indole Ring

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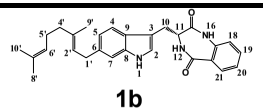
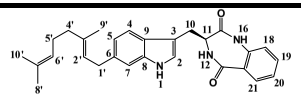
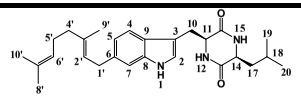
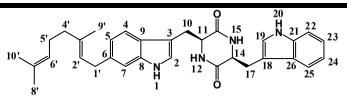
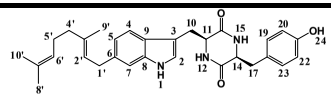
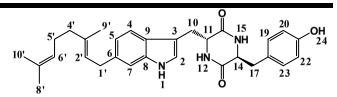
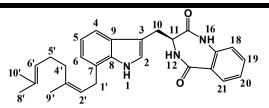
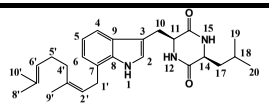
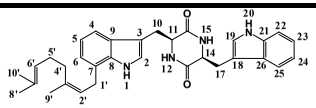
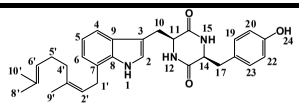
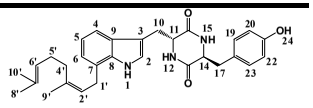
cbic_201300372_sm_miscellaneous_information.pdf

Table S1: ^{1}H-NMR data of C6-geranylated enzyme products (b-series) in $CDCl_3$ (**1b**-**4b**) and DMSO-d6 (**5b** and **6b**).

compd.	 **1b**	 **2b**	 **3b**	 **4b**	 **5b**	 **6b**
pos.	δ_H(ppm), multi., *J* (Hz)	δ_H(ppm), multi., *J* (Hz)	δ_H(ppm), multi., *J* (Hz)	δ_H(ppm), multi., *J* (Hz)	δ_H (ppm), multi., *J* (Hz)	δ_H (ppm), multi., *J* (Hz)
1	7.98, s	7.97, s	8.00, s	8.02, s	8.27, s	8.27, s
2	7.11, d, 2.3	7.11, d, 2.3	7.04, d, 2.3	6.51, s[c]	6.87, d, 2.0	6.89, d, 2.3
4	7.41, d, 8.1	7.41, d, 8.1	7.52, d, 8.1	7.56, d, 8.2	7.34, d, 8.1	7.38, d, 8.1
5	6.79, d, 8.1	6.94, dd, 8.1, 1.3	6.99, dd, 8.1, 1.3	6.99, d, 8.2	6.78, d, 8.1	6.73, dd, 8.1, 1.3
7	7.16, br s	7.17, br s	7.17, br s	7.14, s	7.05, s	7.03, s
10a	3.49, dd, 15.1, 5.6	3.49, dd, 15.2, 5.6	3.47, dd, 15.0, 4.5	3.21, dd, 14.5, 3.5	2.75, dd, 15.0, 4.5	3.05, dd, 14.5, 5.0
10b	3.20, dd, 15.1, 8.5	3.20, dd, 15.2, 8.4	3.14,dd, 15.0, 8.7	2.47, dd, 14.5, 9.0	2.38, dd, 15.0, 6.5	2.83, dd, 14.5, 4.0
11	4.12, dt, 8.5, 5.6	4.12, dt, 8.4, 5.6	4.29, br d, 8.7	4.17, m	4.03, dd, 6.5, 4.5	3.35, m
12	6.28, d, 4.8	6.05, d, 4.9	5.77, s[a]	5.65, s	7.71, d, 2.4[d]	7.75, d, 1.4[e]
14	-	-	3.89, br d, 10.2	4.17, m	3.89, m	3.30, m
15	-	-	5.75, s[a]	5.65, s	7.50, d, 2.4[d]	7.76, d, 1.2[e]
16	8.00, s	7.68, s	-	-	-	-
17a	-	-	1.60, m	3.24, dd, 14.5, 3.5	2.38, dd, 15.0. 6.4	2.80, dd, 14.5, 4.0
17b	-	-	1.02, ddd, 13.8, 10.2, 4.6	2.44, dd, 14.5, 9.0	1.71, dd, 15.0, 6.5	2.56, dd, 14.5, 5.0
18	6.94, d, 8.1	6.95, dd, 8.1, 0.7	1.49, m	-	-	-
19	7.49, td, 7.3, 0.8	7.49, td, 7.7, 1.1	0.84, d, 8.4[b]	6.56, s[c]	6.54, d, 8.5	6.85, d, 8.5
20	7.26, td, 7.3, 0.8	7.25,td, 7.7, 1.1	0.83, d, 8.4[b]	7.92, s	6.45, d, 8.4	6.58, d, 8.5
21	7.90, d, 7.8	7.91, dd, 7.9, 1.5	-	-	-	-
22	-	-	-	7.34, d, 8.0	6.45, d, 8.4	6.58, d, 8.5
23	-	-	-	7.21, t, 7.6	6.54, d, 8.5	6.85, d, 8.5
24	-	-	-	7.14, t, 7.6	10.67, s	10.63, s
25	-	-	-	7.45, d, 7.6	-	-
1'	3.43, d, 7.3	3.44, d, 7.3	3.45, d, 7.3	3.45, d, 7.0	3.35, d, 7.5	3.33, d, 7.5
2'	5.36, td, 7.2, 0.9	5.36, td, 7.3, 1.2	5.37, td, 7.3, 1.2	5.37, t, 7.0	5.31, t, 7.5	5.31, t, 7.5
4'	2.10, m	2.10, m	2.11, m	2.12, m	2.04, m	2.05, dd, 15.0, 7.5
5'	2.04, m	2.04, m	2.05, m	2.05, m	1.98, m	1.98, m
6'	5.10, t, 6.8	5.10, t, 6.9	5.11, t, 6.8	5.11, t, 6.8	5.06, t, 6.3	5.06, t, 6.9
8'	1.72, s	1.72, s	1.73, s	1.73, s	1.67, s	1.67, s
9'	1.67, s	1.67, s	1.68, s	1.67, s	1.60, s	1.60, s
10'	1.59, s	1.59, s	1.60, s	1.59, s	1.52, s	1.53, s

[a-e]assignments with same letters are interchangeable

Table S2: ^{1}H-NMR data of C7-geranylated enzyme products (c-series) in $CDCl_3$ (**2c**-**4c**) and DMSO-d6 (**5c** and **6c**).

compd.	 **2c**	 **3c**	 **4c**	 **5c**	 **6c**
pos.	δ_H(ppm), multi., *J* (Hz)	δ_H(ppm), multi., *J* (Hz)	δ_H(ppm), multi., *J* (Hz)	δ_H(ppm), multi., *J* (Hz)	δ_H(ppm), multi., *J* (Hz)
1	8.10, s	8.16, s	8.05, s	9.08, s	9.16, s
2	7.16, d, 2.3	7.07, s	6.53, d, 2.2[c]	6.94, d, 2.1	6.96, d, 2.3
4	7.38, dd, 7.5, 0.8	7.49, d, 7.7	7.41, d, 8.2	7.27, d, 7.7	7.32, d, 7.8
5	7.04, t, 7.5	7.07, t, 7.7	7.07, t, 7.2	6.87, t, 7.7	6.83, t, 7.8
6	7.01, dd, 7.1, 1.2	7.03, d, 7.1	7.03, d, 7.1	6.81, d, 7.0	6.77, d, 7.2
10a	3.51, dd, 15.5, 5.6	3.48, dd, 14.6, 3.7	3.23, dd, 14.5, 1.9	2.76, dd, 14.0, 4.0	3.07, dd, 14.5, 4.1
10b	3.23, dd, 15.5, 8.3	3.17, dd, 14.6, 8.5	2.39, dd, 14.5, 8.7	2.39, dd, 14.0, 4.4	2.83, dd, 14.5, 5.2
11	4.13, dt, 8.3, 5.6	4.30, d, 8.5	4.18, t, 8.7	3.89, m	3.39, m
12	6.15, d, 4.9	6.21, s[a]	5.75, s[d]	7.72, d, 2.4	7.77, d, 1.3
14	-	3.88, d, 10.1	4.18, t, 8.7	3.75, m	3.30, m
15	-	6.04, s[a]	5.74, s[d]	7.54, d, 2.5	7.76, d, 1.5
16	7.82, s	-	-	-	-
17a	-	1.58, dd, 14.0, 4.0	3.23, dd, 14.5, 1.9	2.39, dd, 14.0, 4.4	3.22*, m
17b	-	0.98, m	2.50, dd, 14.5, 8.7	1.79, dd, 14.0, 6.9	2.60, m
18	6.96, dd, 8.1, 0.7	1.51, m	-	-	-
19	7.49, td, 8.1, 1.0	0.83, d, 6.3[b]	6.58, d, 2.2[c]	6.55, d, 8.6	6.85, d, 8.5
20	7.25, td, 7.7, 1.0	0.81, d, 6.3[b]	8.05, s	6.50, d, 8.6	6.58, d, 8.5
21	7.90, dd, 7.7, 1.5	-	-	-	-
22	-	-	7.35, d, 8.2	6.50, d, 8.6	6.58, d, 8.5
23	-	-	7.22, td, 8.2, 1.1	6.55, d, 8.6	6.85, d, 8.5
24	-	-	7.15, td, 8.1, 1.0	10.77, s	10.73, s
25	-	-	7.57, d, 8.1	-	-
1'	3.56, d, 7.0	3.56, d, 7.0	3.53, d, 6.8	3.45, d, 7.5	3.47, d, 7.0
2'	5.41, td, 7.0, 1.1	5.41, td, 7.0, 1.1	5.32, td, 6.8, 1.1	5.32, td, 7.5, 1.2	5.38, td, 7.7, 1.2
4'	2.12, m	2.13, m	2.08, m	1.98, m	2.05, m
5'	2.09, d, 6.2	2.09, m	2.02, m	1.90, m	1.99, m
6'	5.09, t, 7.3	5.09, t, 7.4	5.05, t, 6.7	5.02, td, 7.0, 1.5	5.06, t, 7.6
8'	1.79, s	1.79, s	1.76, s	1.62, s	1.66, s
9'	1.68, s	1.68, s	1.68, s	1.60, s	1.60, s
10'	1.59, s	1.60, s	1.57, s	1.51, s	1.52, s

[a-d] assignments with same letters are interchangeable

*overlapping with solvent signal

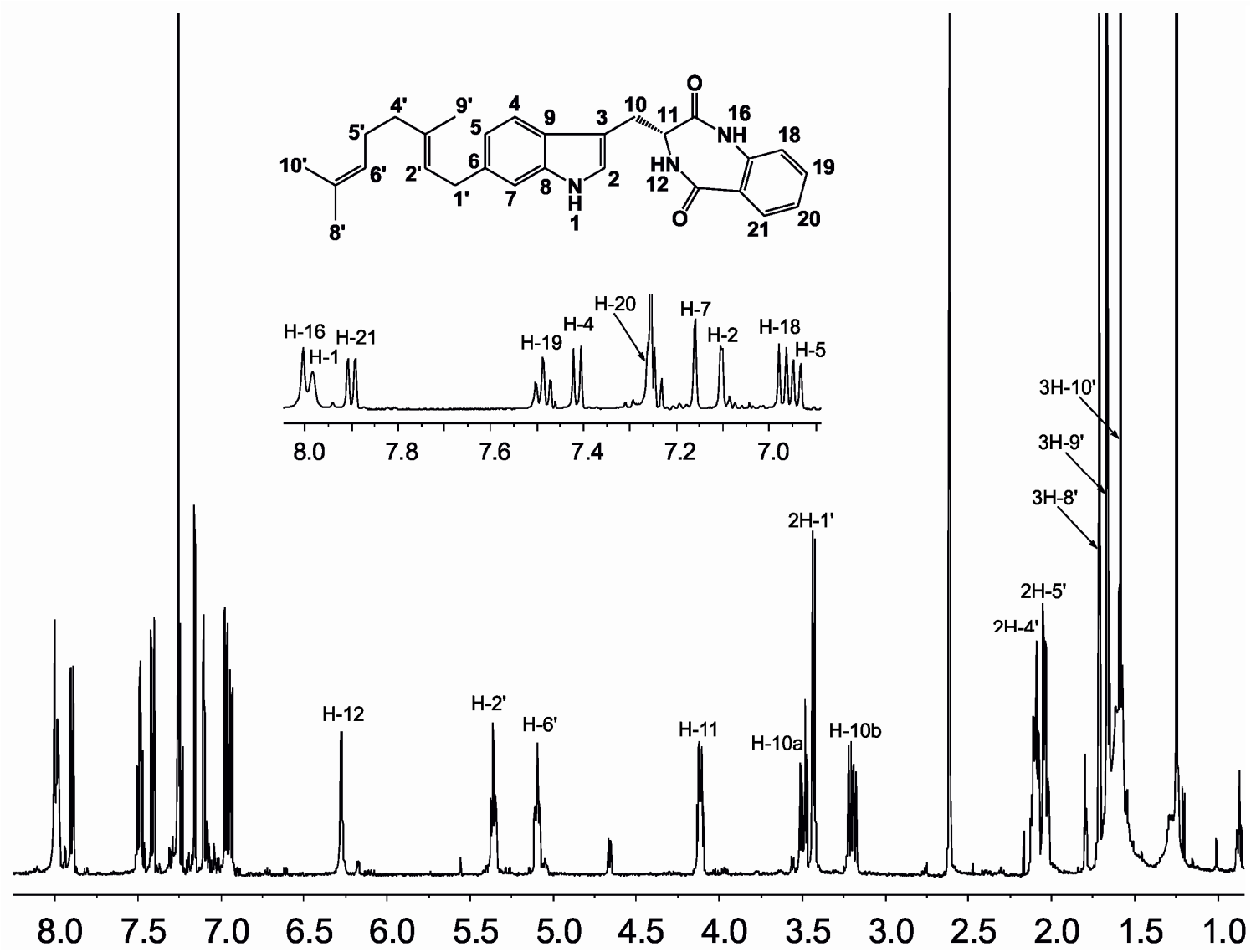


Figure S1: ^{1}H-NMR spectrum of **1b** in $CDCl_3$.

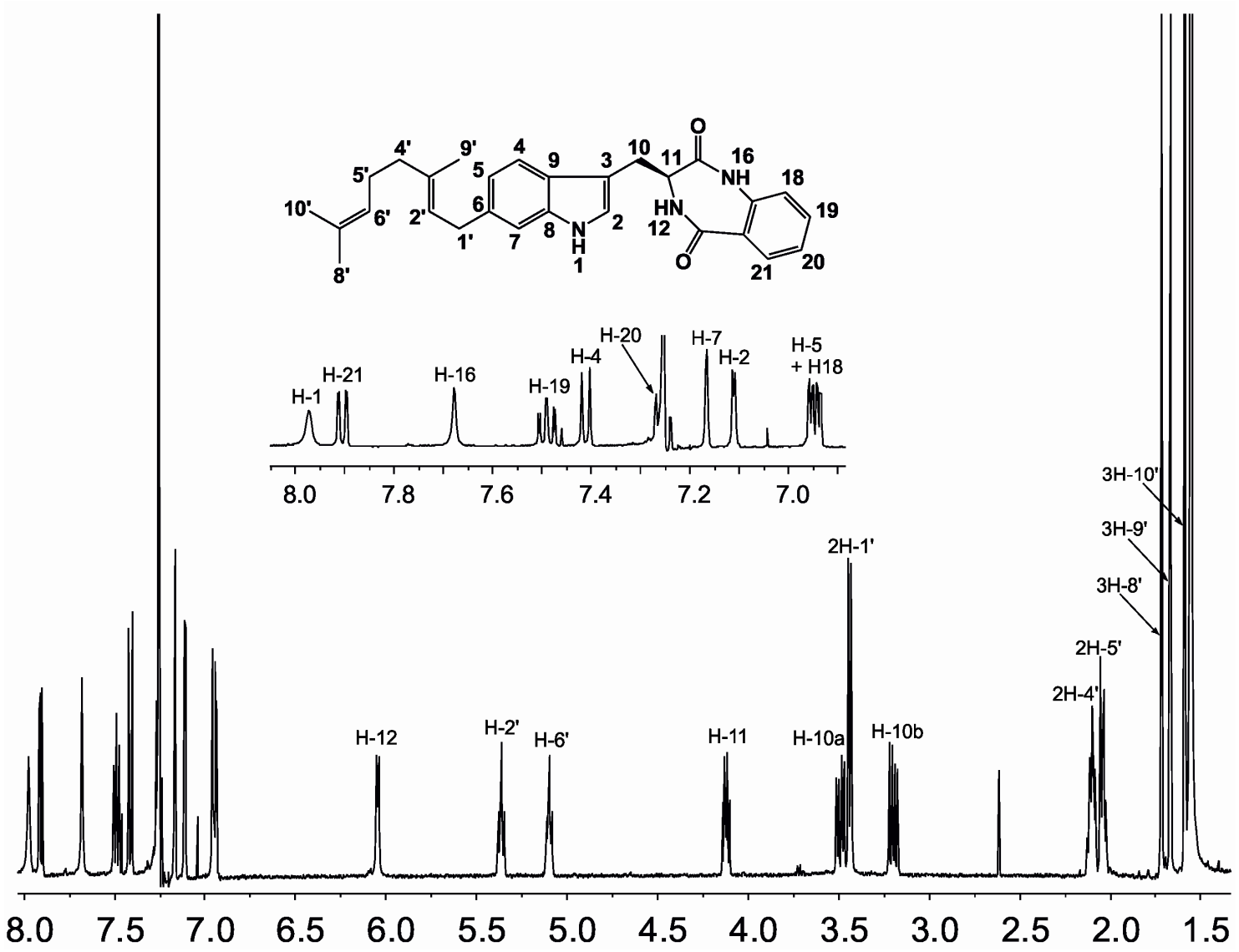


Figure S2: ^{1}H-NMR spectrum of **2b** in $CDCl_3$.

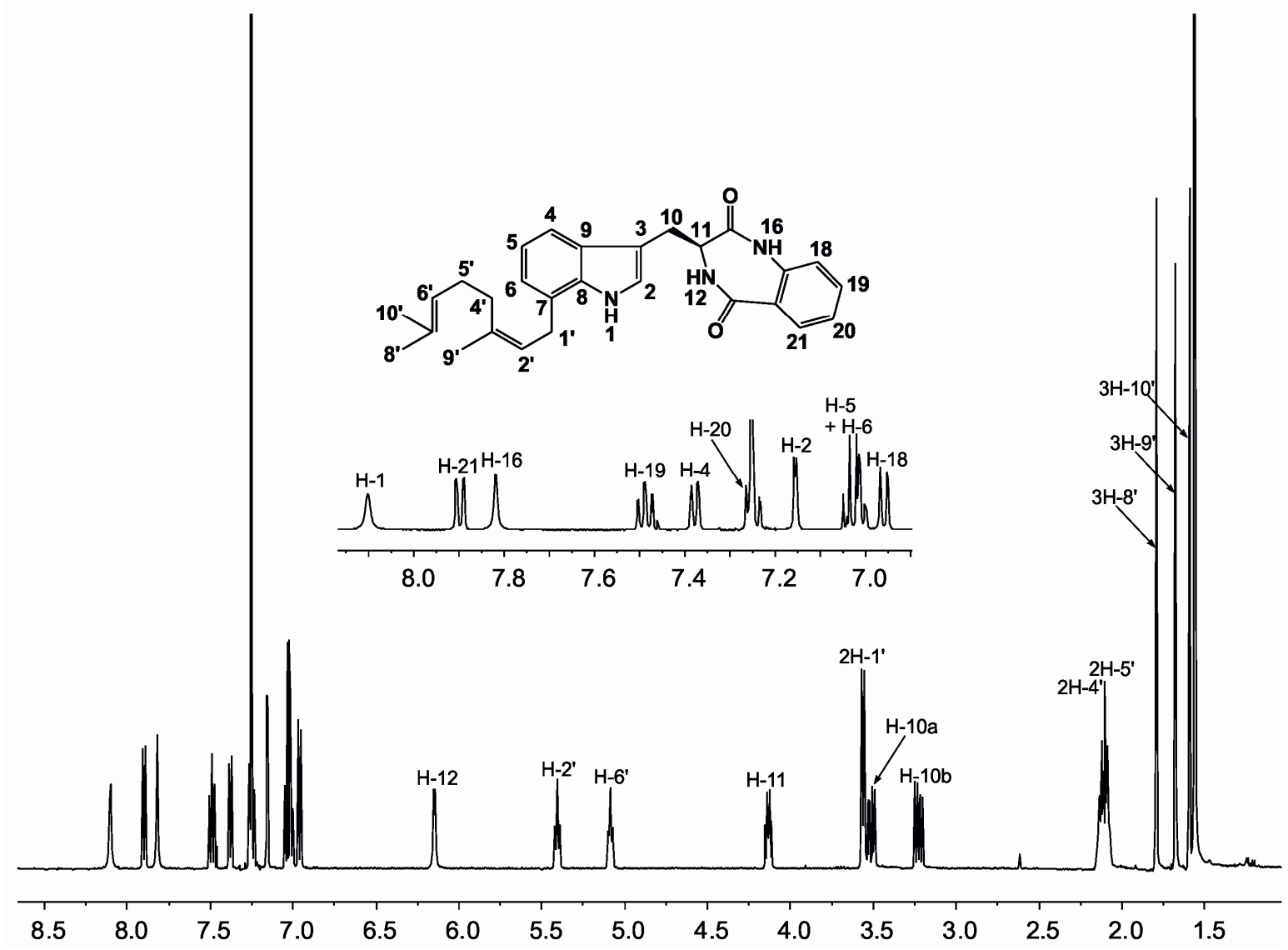


Figure S3: ^{1}H-NMR spectrum of **2c** in $CDCl_3$.

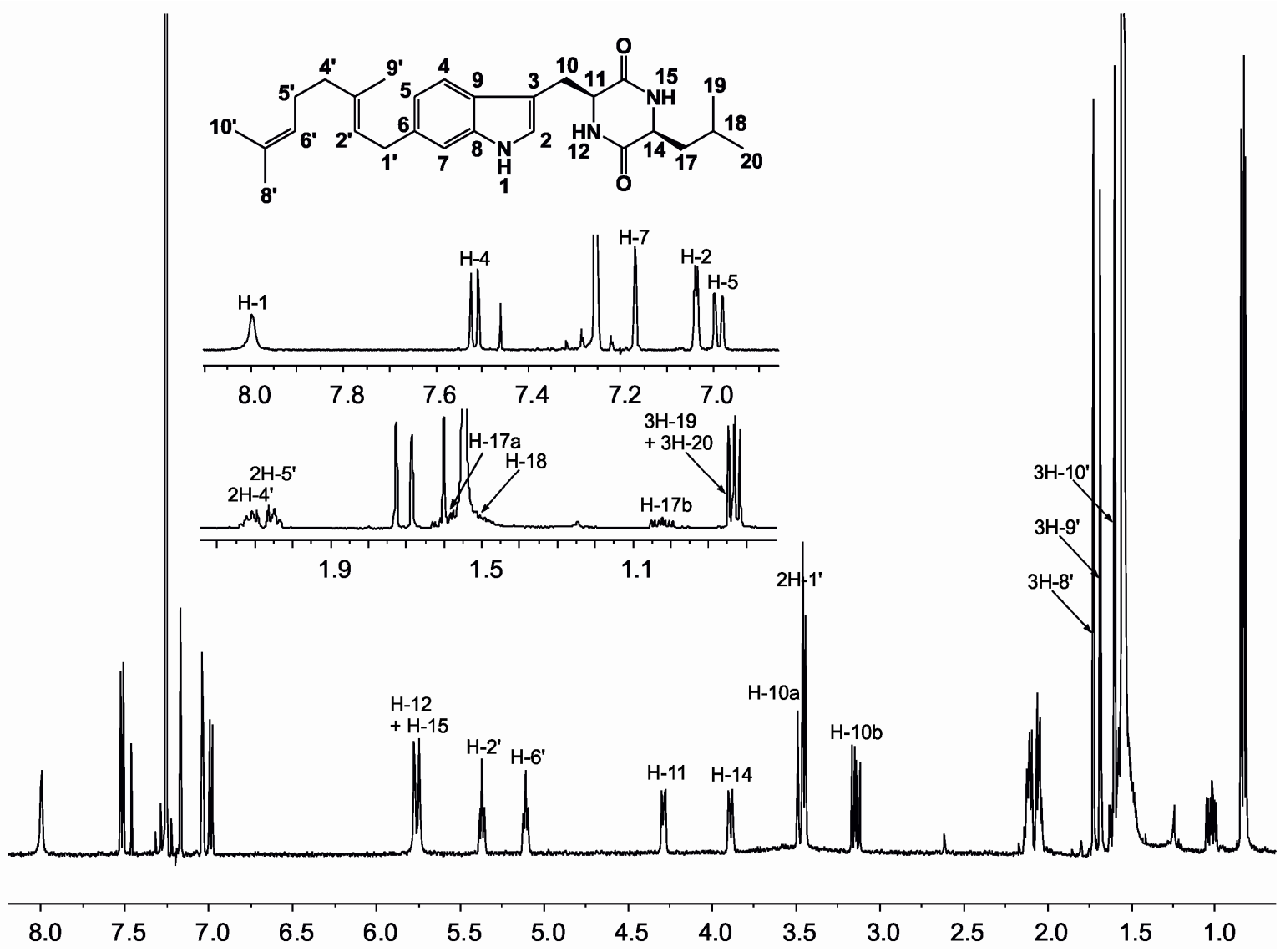


Figure S4: ^{1}H-NMR spectrum of **3b** in $CDCl_3$.

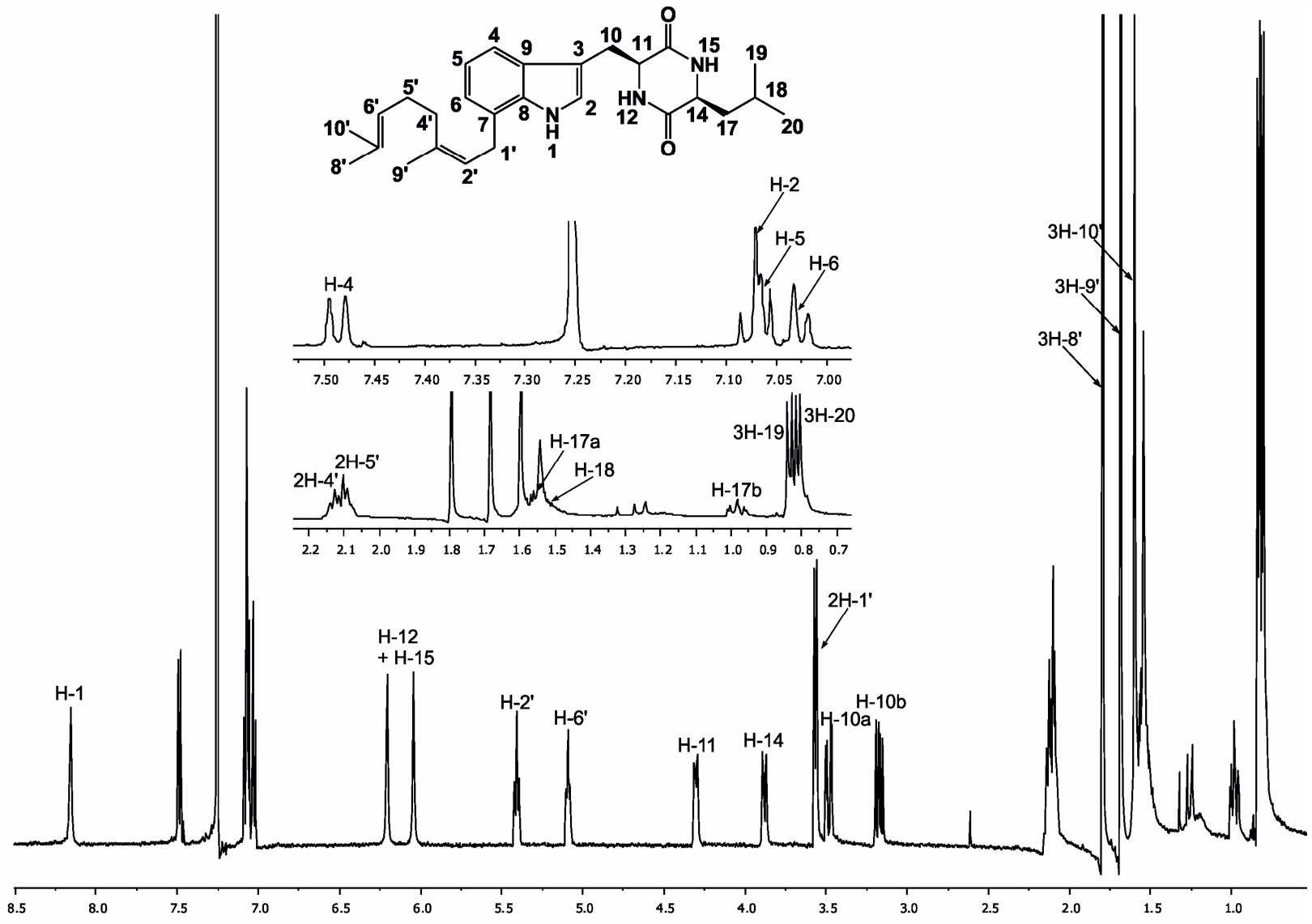


Figure S5: ^{1}H-NMR spectrum of **3c** in $CDCl_3$.

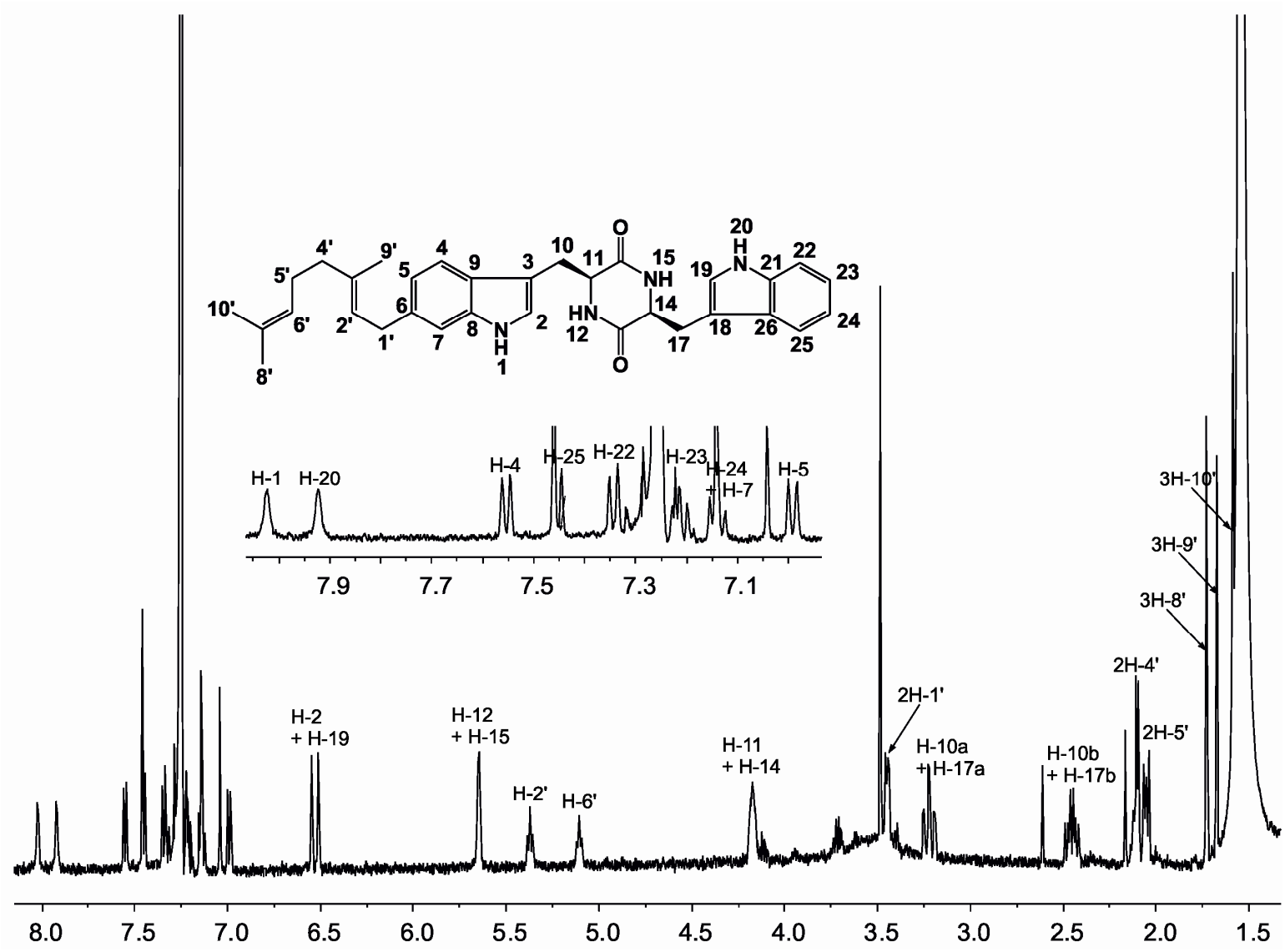


Figure S6: ^{1}H-NMR spectrum of **4b** in $CDCl_3$.

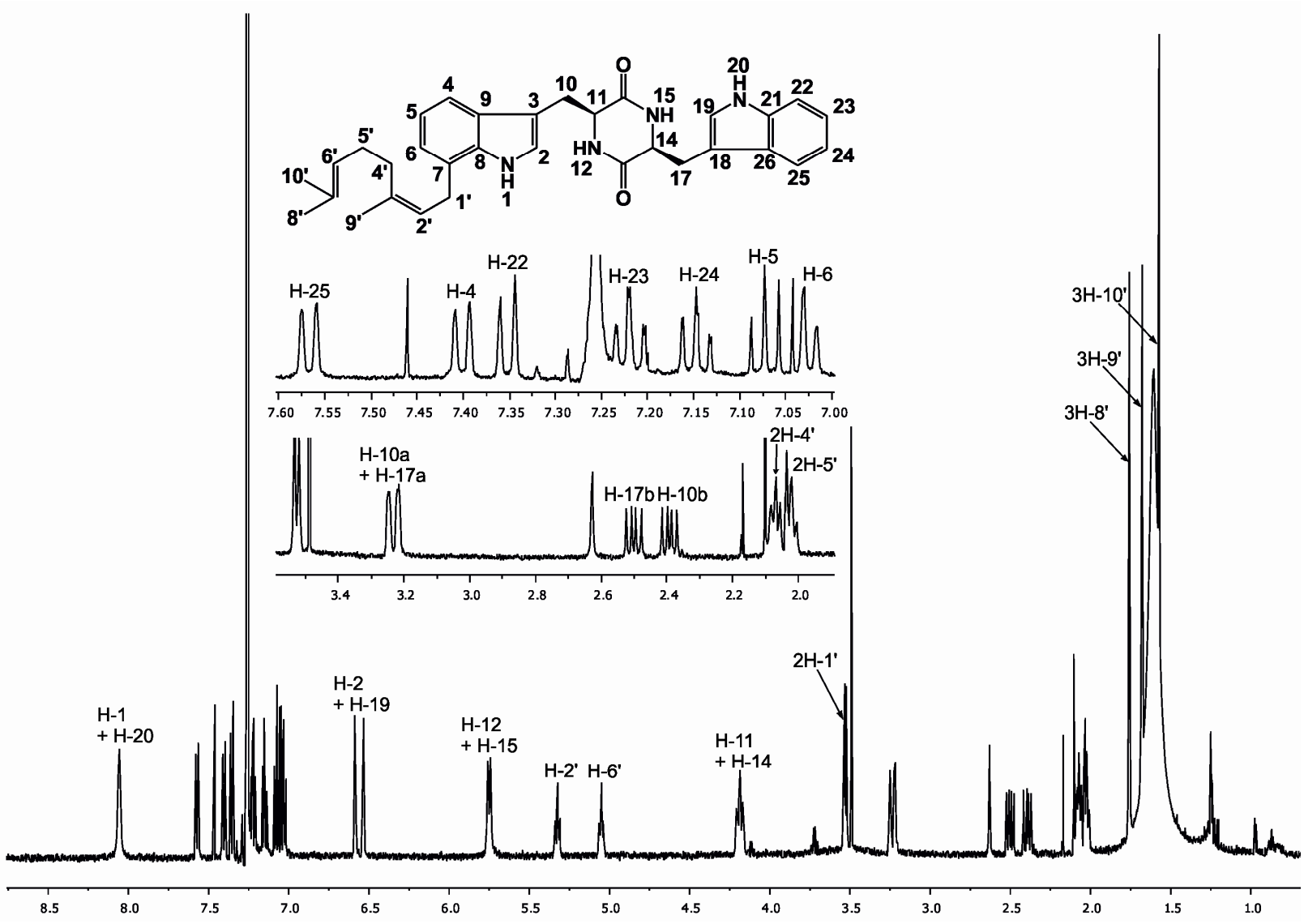


Figure S7: ^{1}H-NMR spectrum of **4c** in $CDCl_3$.

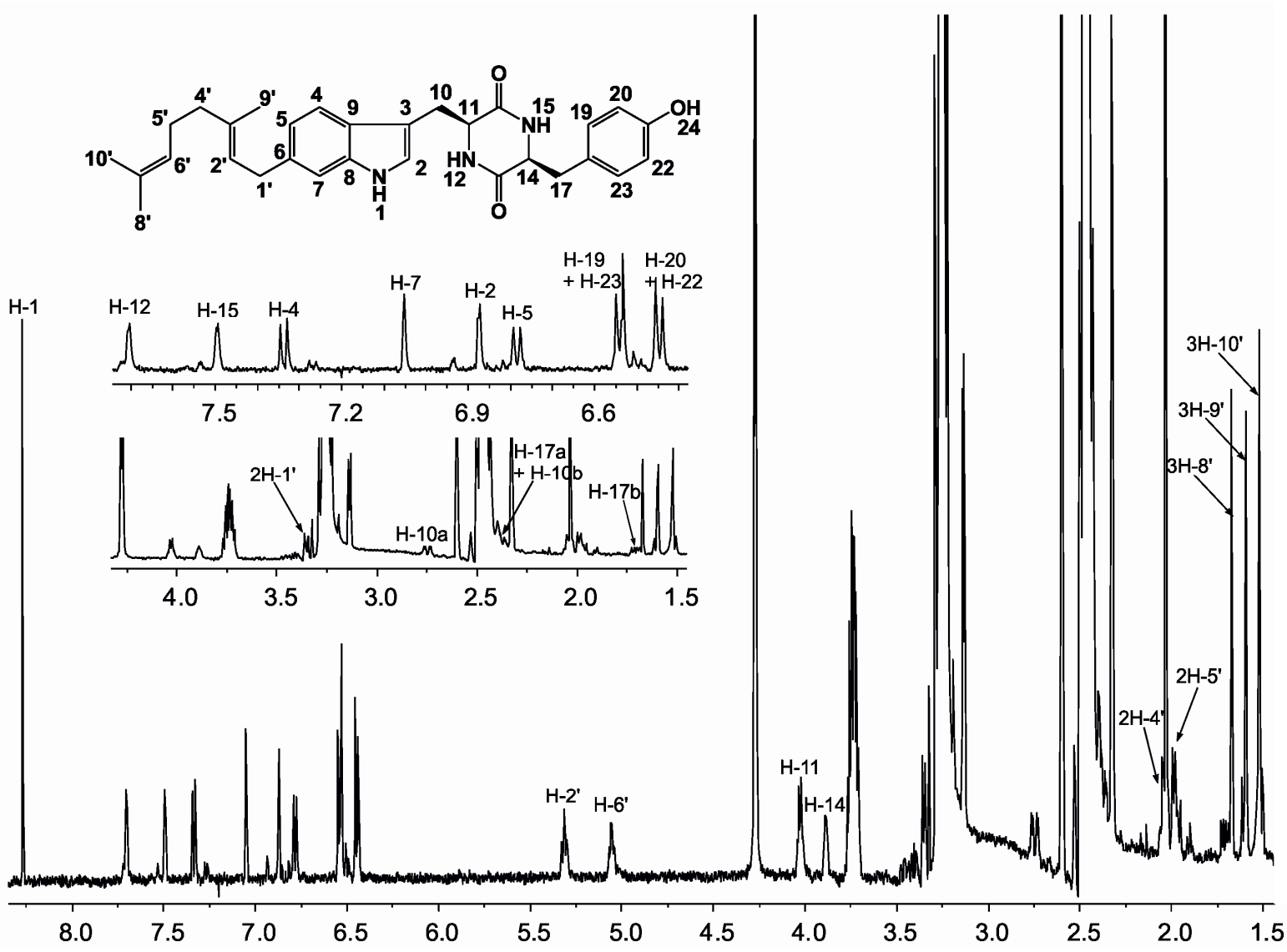


Figure S8: ^{1}H-NMR spectrum of **5b** in DMSO-d6.

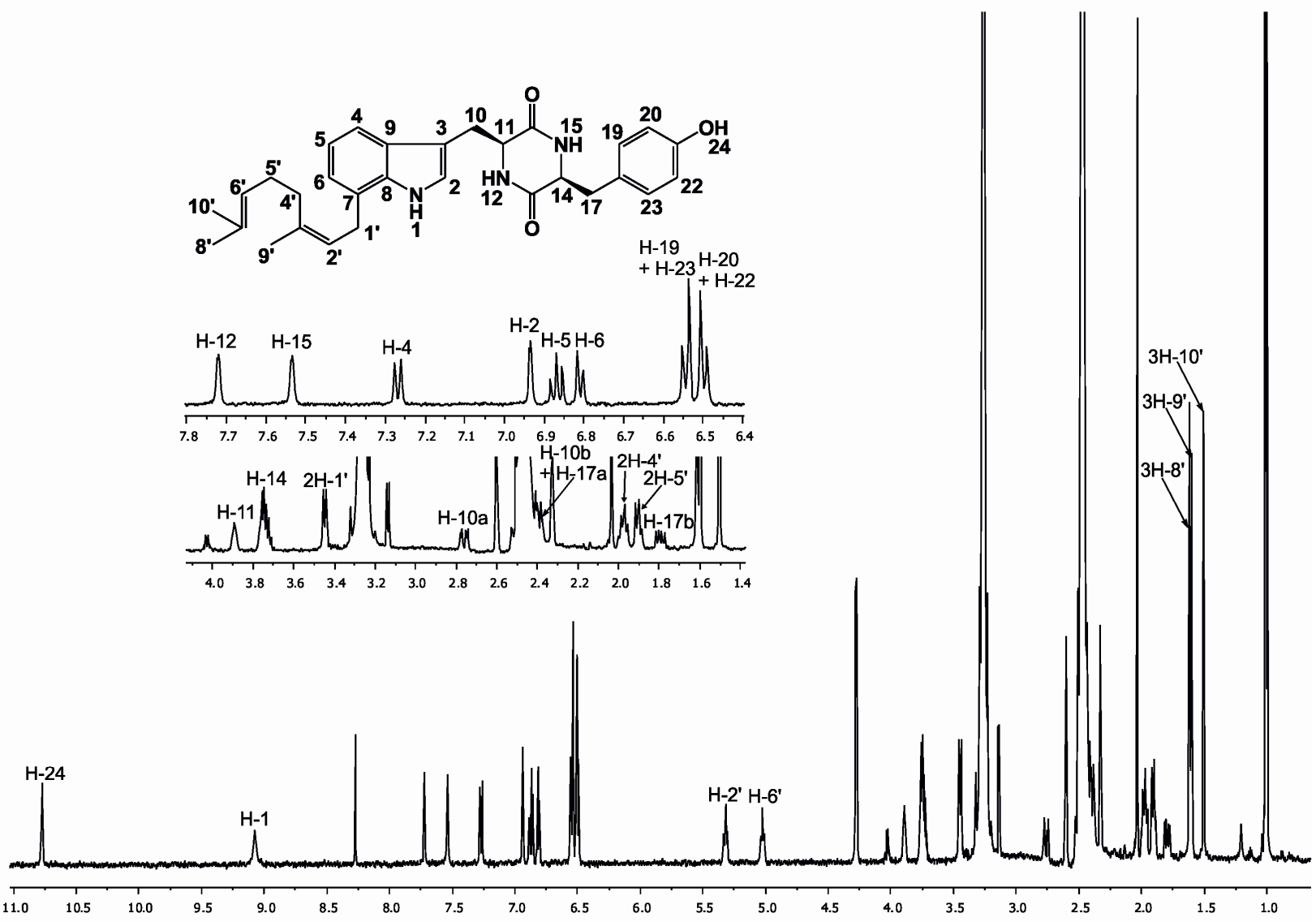


Figure S9: ^{1}H-NMR spectrum of **5c** in DMSO-d6.

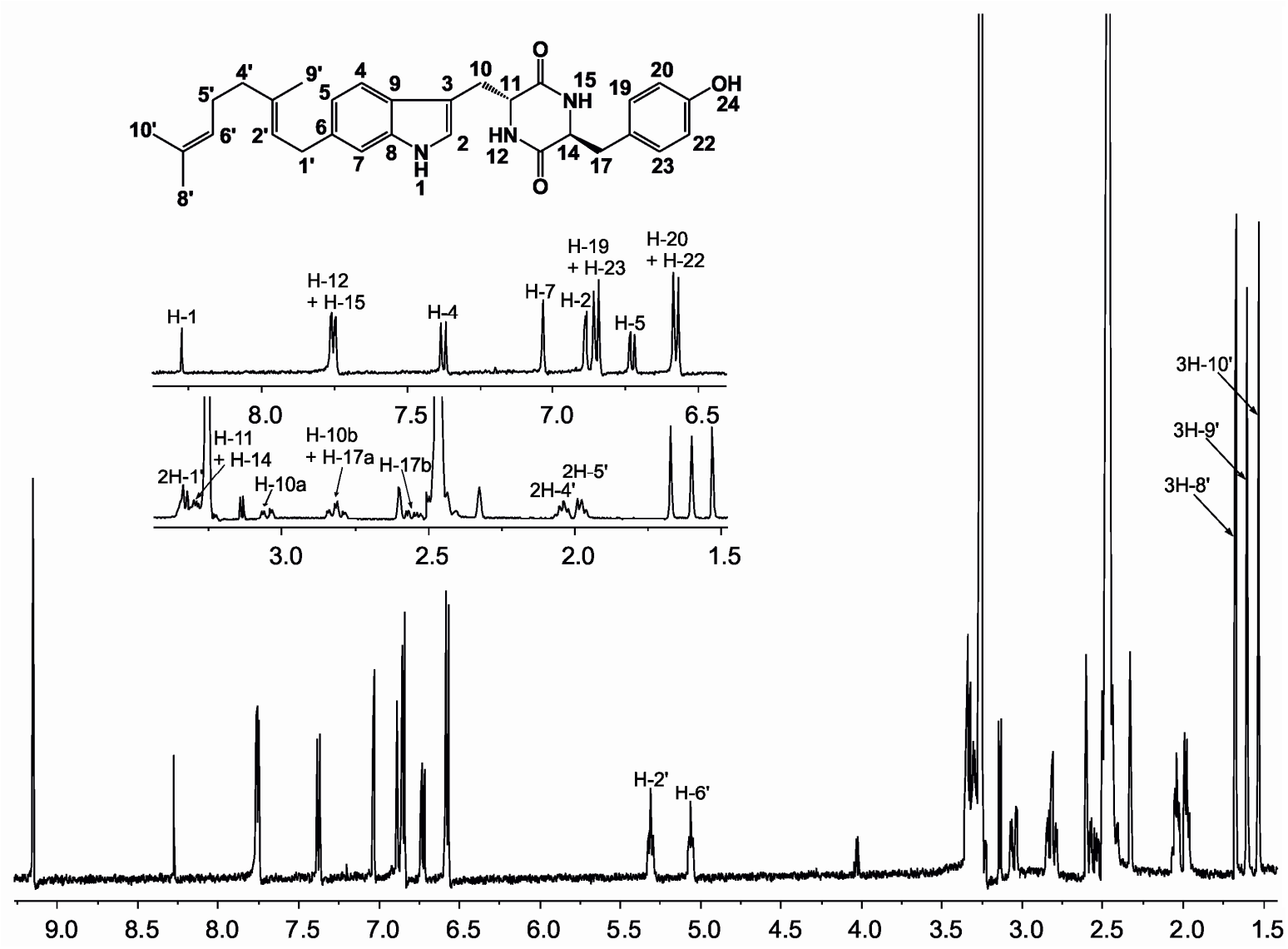


Figure S10: ^{1}H-NMR spectrum of **6b** in DMSO-d6.

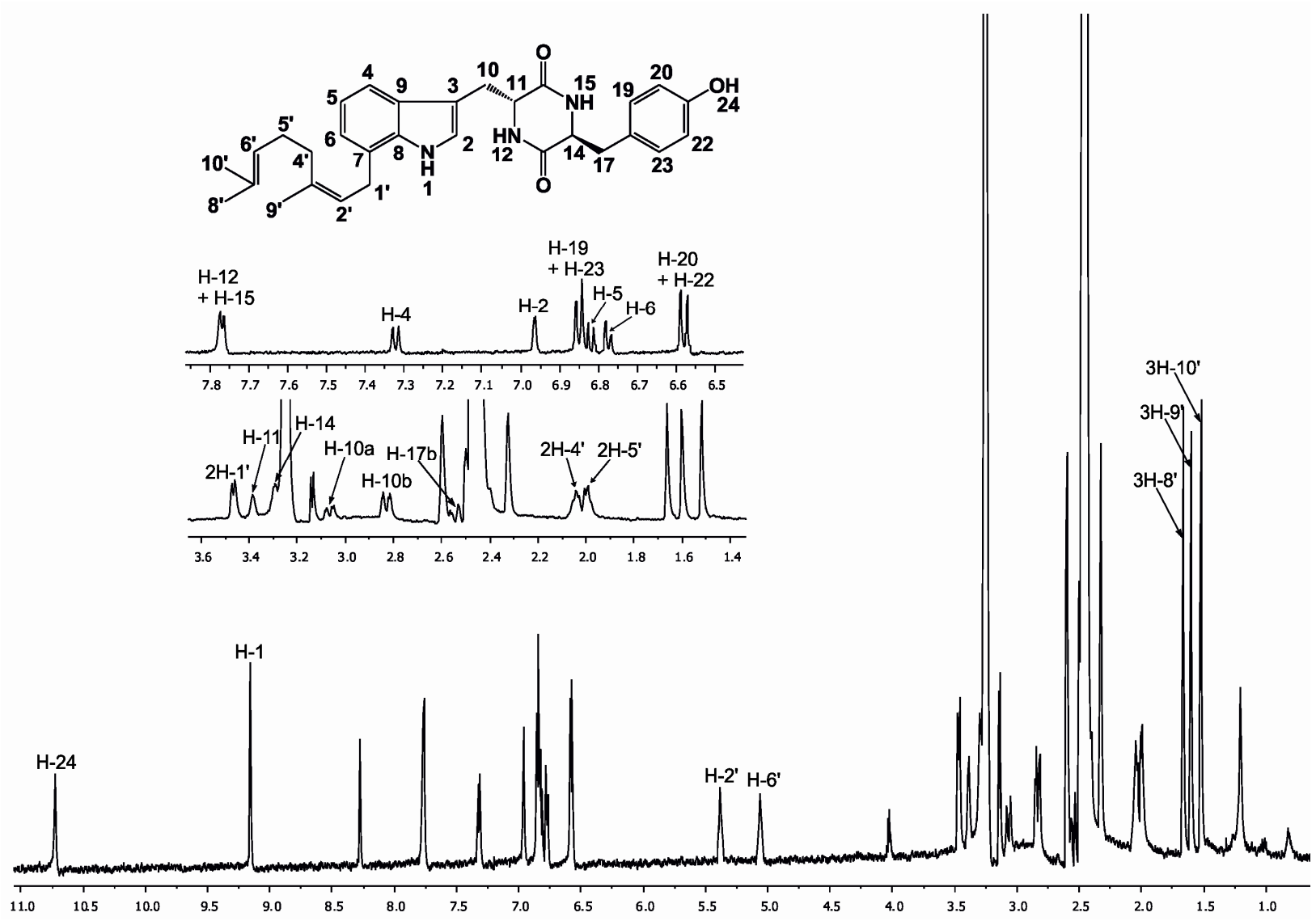


Figure S11: ^{1}H-NMR spectrum of **6c** in DMSO-d6.

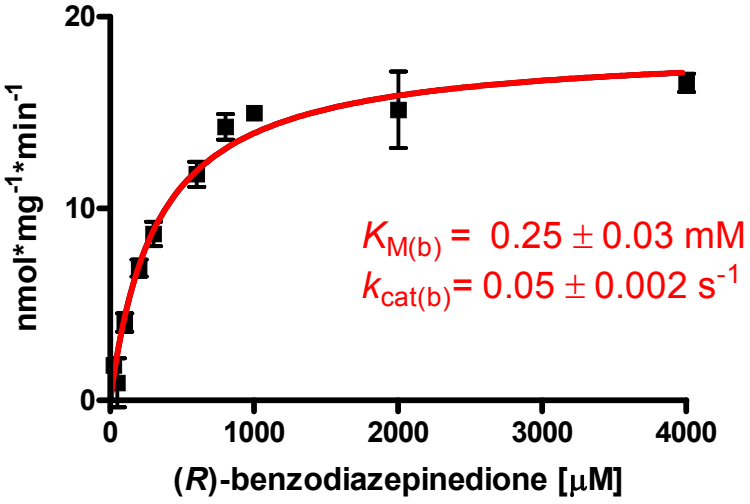


Figure S12: Determination of kinetic parameters for AnaPT with (*R*)-benzodiazepinedione (**1a**).

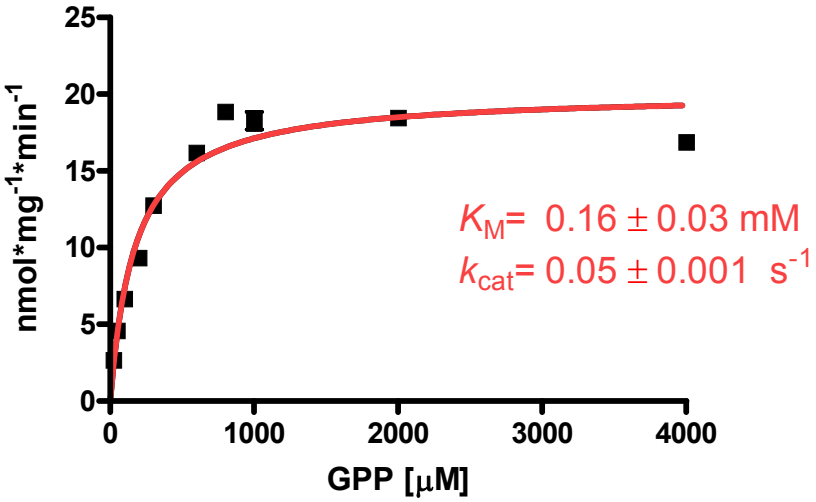


Figure S13: Determination of kinetic parameters for AnaPT with GPP using **1a** as aromatic substrate.

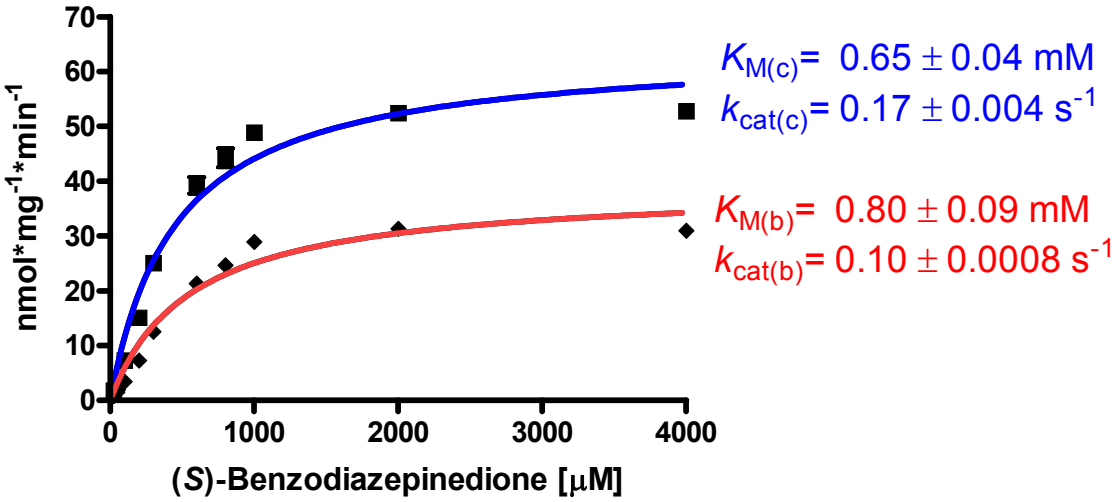


Figure S14: Determination of kinetic parameters for AnaPT with (*S*)-benzodiazepinedione (**2a**).

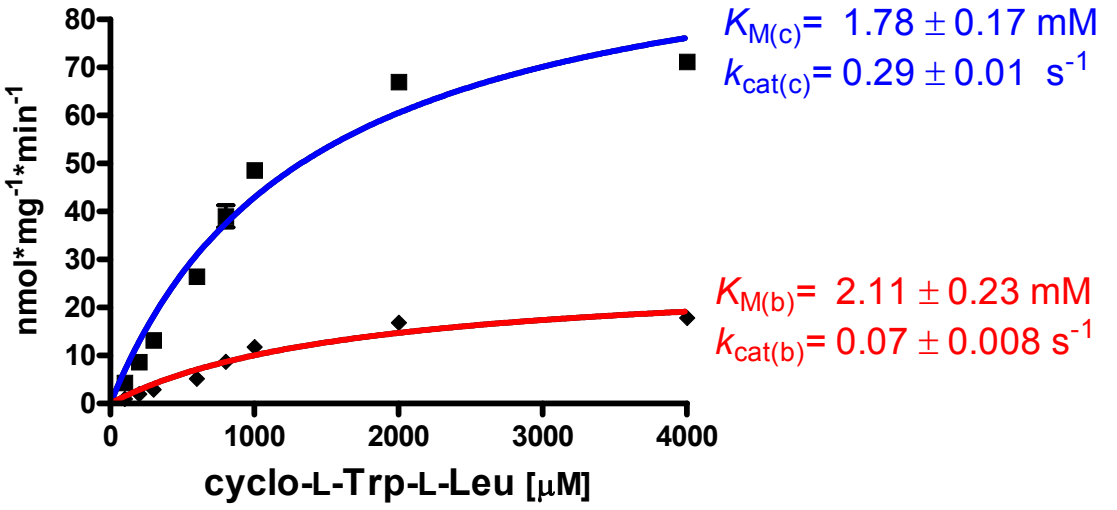


Figure S15: Determination of kinetic parameters for AnaPT with cyclo-L-Trp-L-Leu (**3a**).

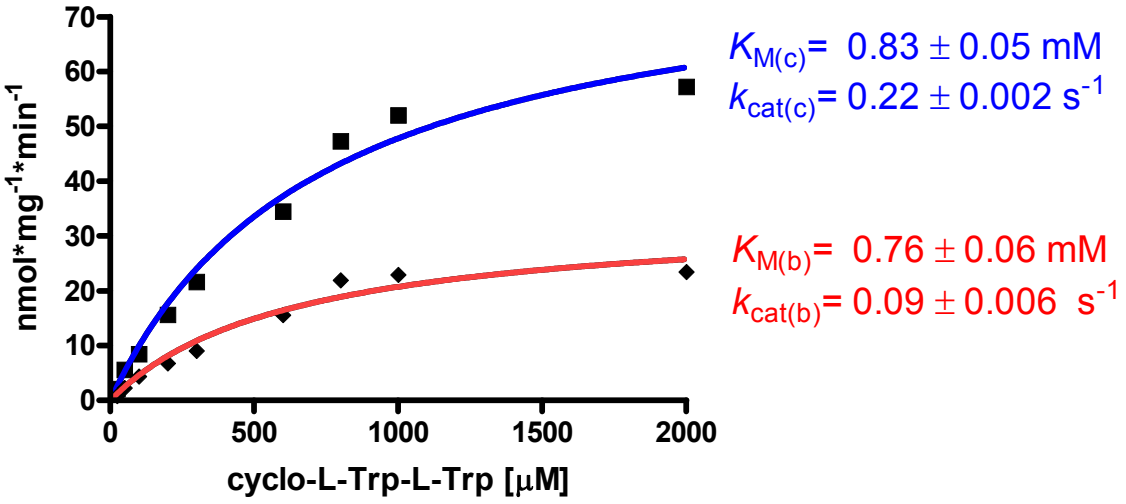


Figure S16: Determination of kinetic parameters for AnaPT with cyclo-L-Trp-L-Trp (**4a**).

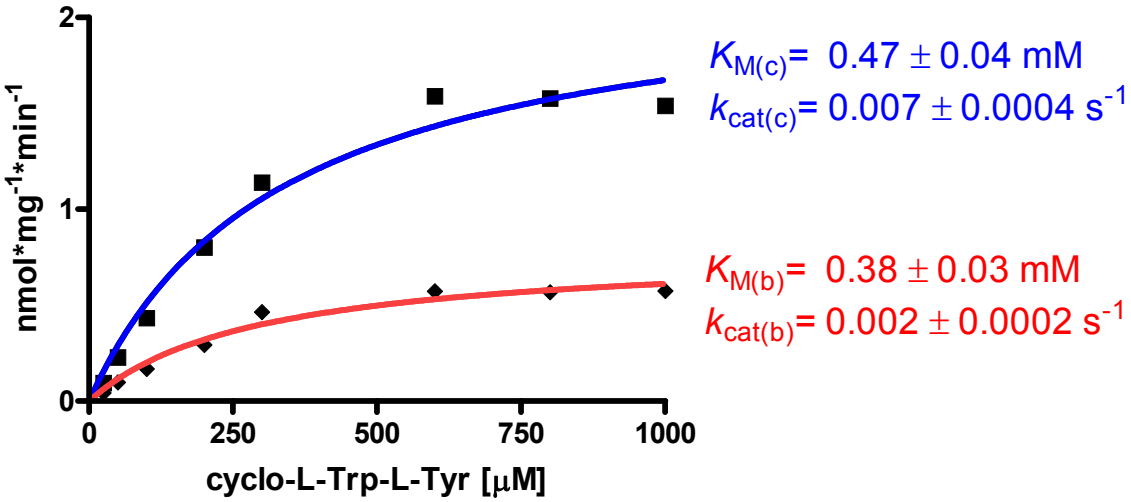


Figure S17: Determination of kinetic parameters for AnaPT with cyclo-L-Trp-L-Tyr (**5a**).

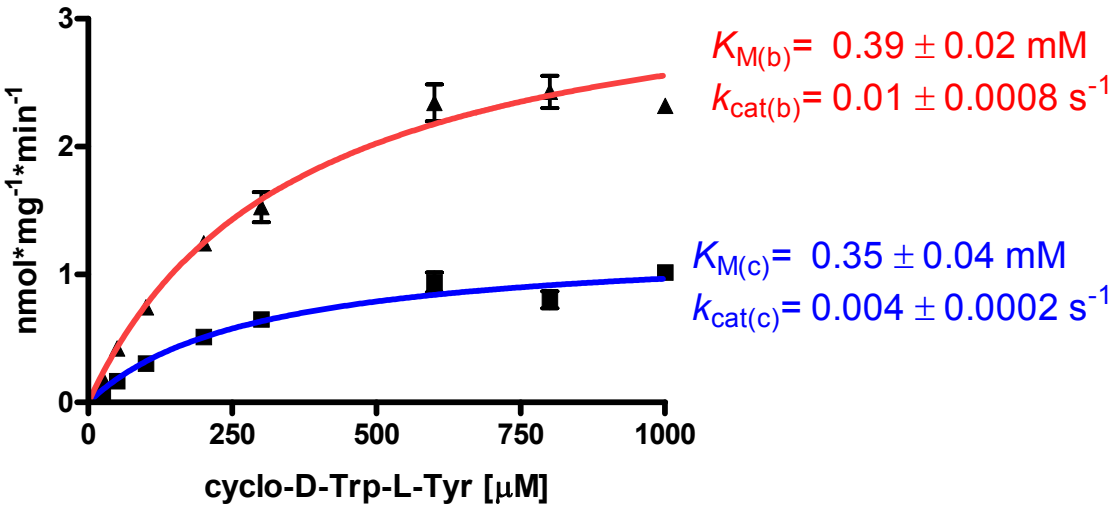


Figure S18: Determination of kinetic parameters for AnaPT with cyclo-D-Trp-L-Tyr (**6a**).

4.3 A Promiscuous Prenyltransferase from *Aspergillus oryzae* catalyses C-prenylations of hydroxynaphthalenes in the presence of different prenyl donors

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A promiscuous prenyltransferase from *Aspergillus oryzae* catalyses C-prenylations of hydroxynaphthalenes in the presence of different prenyl donors

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Abstract Prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily are involved in the biosynthesis of secondary metabolites and show broad substrate specificity towards their aromatic substrates with a high regioselectivity for the prenylation reactions. Most members of this superfamily accepted as prenyl donor exclusively dimethylallyl diphosphate (DMAPP). One enzyme, AnaPT from *Neosartorya fischeri*, was reported recently to use both DMAPP and geranyl diphosphate (GPP) as prenyl donors. In this study, we demonstrate the acceptance of DMAPP, GPP and farnesyl diphosphate (FPP) by a new member of this superfamily, BAE61387 from *Aspergillus oryzae* DSM1147, for C-prenylations of hydroxynaphthalenes.

Keywords DMATS superfamily · Prenyl donor · Hydroxynaphthalene · Enzyme catalysis

Introduction

Prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily are mainly found in fungi of ascomycetes and involved in secondary metabolite biosynthesis. They catalyse transfer reactions of a prenyl moiety (n×C5 units), usually from prenyl diphosphate, e.g. dimethylallyl diphosphate (DMAPP), onto aromatic ring systems of diverse substrates (Yu and Li 2012). Prenylation usually increases the lipophilicity of the resulted products and strengthens their interaction with proteins and biomembranes and makes these enzymes interesting for pharmaceutical and medicinal research (Botta et al. 2005).

Molecular biological and biochemical investigations with genes/enzymes from this superfamily in recent years have revealed that these enzymes displayed much higher flexibility towards their aromatic substrates than our knowledge 5 years ago. At that time, substrates of known prenyltransferases from this family were exclusively indole derivatives (Steffan et al. 2009). It is known today that several enzymes from this group use tyrosine or nitrogen-free aromatic compounds such as xanthones, anthracene or naphthacene derivatives as natural substrates (Chooi et al. 2012, 2013; Kremer and Li 2010; Pockrandt et al. 2012; Sanchez et al. 2011). Biochemical investigations have also revealed that several members from this superfamily used distinctly different compounds from their natural substrates and catalysed regiospecific prenylations. These include hydroxynaphthalenes (Yu et al. 2011), flavonoids (Yu and Li 2011) and tyrosine derivatives (Zou et al. 2011).

The feature of relaxed substrate specificity of the prenyltransferases was successfully used in chemoenzymatic synthesis for the production of prenylated derivatives (Yu and Li 2012). On the other hand, most of these enzymes are restricted to the common prenyl donor DMAPP (C_5-unit). VrtC from *Penicillium aethiopicum* utilizes geranyl diphosphate (GPP) (C_{10}-unit), but not DMAPP as prenyl donor (Chooi et al. 2010). We have very recently demonstrated the acceptance of GPP by the dimethylallyltransferase AnaPT from *Neosartorya fischeri* and the regiospecific geranylation of tryptophan-containing cyclic dipeptides (Pockrandt and Li 2013). These results provided experimental evidence for the relationship of DMAPP- and GPP-using enzymes from this family. We

C. Sack and T. Kosiol contributed to this manuscript as part of their practical training course.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-5509-x) contains supplementary material, which is available to authorized users.

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speculated that some members of the DMATS superfamily could be even capable of utilizing DMAPP, GPP and farnesyl diphosphate (C_{15}-unit, FPP) as prenyl donors (Pockrandt and Li 2013). However, none of the known enzymes so far accepted FPP as prenyl donor. We took the new challenge of searching for prenyltransferases with broad substrate specificity towards their prenyl donors. Here, we report the biochemical characterization of such an enzyme BAE61387 from *Aspergillus oryzae* DSM1147 for prenylation of hydroxynaphthalenes.

Materials and methods

Computer-assisted sequence analysis

The software FGENESH (Softberry, Inc; http://www.softberry.com/berry.phtml) was used for prediction of exon and intron sequences. Alignments of amino acid sequences were carried out by using the program BLAST (http://blast.ncbi.nlm.nih.gov).

Chemicals

DMAPP, GPP and FPP were synthesized as trisammonium salts according to the method described for GPP trisammonium (Woodside et al. 1988). Naphthalene derivatives were purchased in the highest available purity from Fluka, TCI and Acros Organics.

Bacterial strains, plasmids and culture conditions

The expression vector pQE60 was obtained from Qiagen (Hilden, Germany). *Escherichia coli* strain XL1 Blue MRF′ (Stratagene, Amsterdam, the Netherlands) was used for cloning and gene expression. Bacteria harbouring the vectors were grown in liquid or on solid lysogeny broth (LB) media supplemented with agarose (1.5 %*w*/*v*) at 37 °C. Carbenicillin (50 μg mL^{-1}) was used for selection of recombinant *E. coli* strains.

DNA isolation and cloning

Standard procedures for DNA isolation and cloning were performed as described elsewhere (Sambrook and Russell 2001). Genomic DNA for PCR amplification was isolated from *A. oryzae* strain DSM1147 by using phenol/chloroform extraction. The coding sequence of AO090102000322 was amplified using the primer pair BAE61387_*Nco*I (5′-GAA**CCATGG**CTCTTCGGAACG-3′) and BAE61387_*Bgl*II (5′-GA**AGATCT**AACATCAACTGTT-3′). Bold letters represent introduced restriction sites for subsequent cloning. The resulting PCR product was cloned into pGEM-T Easy (Promega) to give the plasmid pDP003, which was sequenced to confirm sequence integrity. In order to create an expression construct, the coding sequence was excised from pDP003 by digestion with *Nco*I and *Bgl*II. The *Nco*I-*Bgl*II fragment of 1,346 bp was isolated from agarose gel and ligated into pQE60 that had been previously digested with *Nco*I and *Bgl*II, yielding the expression plasmid pDP004.

Overproduction and purification of BAE61387-His_6

For gene expression, *E. coli* XL1 Blue MRF′ cells harbouring plasmid pDP004 were cultivated in 2,000-mL Erlenmeyer flasks containing liquid LB media (1,000 mL) supplemented with carbenicillin (50 μg mL^{-1}) at 37 °C and 220 rpm to A_{600}=0.6. For gene induction, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. The bacteria were cultivated for a further 16 h and centrifuged to harvest the cells, which were then resuspended in lysis buffer (imidazole (10 mM), NaCl (300 mM), NaH_2PO_4/Na_2HPO_4 (50 mM, pH 8.0)) at 1.4 mL/g of pellet. After addition of the lysozyme (1 mg mL^{-1}) and incubation on ice for 30 min, the cells were sonicated (6× 10 s, 200 W). To separate cellular debris from soluble proteins, the lysate was centrifuged (14,000×*g*, 30 min, 4 °C). One-step purification of the recombinant BAE61387-His_6 by affinity chromatography with Ni-NTA agarose resin (Qiagen) was carried out according to the manufacturer's instruction. Proteins were eluted with elution buffer (imidazole (250 mM), NaCl (300 mM), NaH_2PO_4/Na_2HPO_4 (50 mM, pH 8.0)). For buffer exchange, the protein fractions were passed through PD-10 columns (GE Healthcare, Freiburg, Germany) that had been equilibrated with Tris-HCl (50 mM, pH 7.5) and glycerol (15 %*v*/*v*). The proteins were eluted with the same buffer, frozen in liquid nitrogen and stored at −80 °C until use. The protein fractions obtained from the purification process were analysed on SDS-PAGE according to the method of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250. The molecular mass of the recombinant BAE61387-His_6 was determined on a HiLoad 16/60 Superdex 200 column (GE Healthcare, Freiburg, Germany) by using 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl as elution buffer. The column was calibrated with dextran blue 2000 (2,000 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa) (GE Healthcare).

Enzyme assays

To determine the relative activity, enzyme assays (100 μL) contained Tris-HCl buffer (50 mM, pH 7.5), aromatic substrate (1.0 mM), DMAPP, GPP or FPP (2 mM), BAE61387 (40 μg), $CaCl_2$ (5 mM), glycerol (3 %*v*/*v*) and dimethyl sulfoxide (DMSO; 2 %*v*/*v*). The reaction mixtures were

incubated at 37 °C with mild agitation on a shaker for 16 h unless otherwise stated. After incubation, enzyme assays were extracted twice with two volumes of ethyl acetate, and the combined organic phases were evaporated to dryness in a vacuum centrifuge. The residue was dissolved in chloroform/methanol (4:1) and analysed by HPLC. For structure elucidation, enzyme assays were scaled up to 40 mL. The reaction mixtures contained Tris-HCl buffer (50 mM, pH 7.5), hydroxynaphthalenes (500 mM), DMAPP, GPP or FPP (1 mM), $CaCl_2$ (5 mM) and BAE61387 (6 mg) were incubated at 37 °C for 16 h. The assays for determination of the kinetic parameters of hydroxynaphthalenes contained DMAPP (2 mM), BAE61387 (40 μg), $CaCl_2$ (5 mM) and hydroxynaphthalenes (0.1–6.0 mM). Assays were incubated at 37 °C for 60 min. For determination of the kinetic parameters of DMAPP, GPP and FPP, the same dilution steps for the prenyl donors were used, **2a** at 1.0 mM was used as aromatic substrate.

HPLC analysis

Enzyme assays were analysed on an Agilent Series 1200 HPLC (Agilent, Foster City, CA) with a MultoHigh 100 Si column (250×4 mm, 5 μm; CS Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 mL min^{-1} with chloroform (solvent A) and methanol (solvent B) as elution solvents. The reaction mixtures were separated with a linear gradient of 0–10 % B for 30 min. After washing with 50 % B for 5 min, the column was equilibrated with 100 % A for 5 min. The substances were detected with a photodiode array detector and illustrated for absorption at 254 nm in this study. For isolation of the enzyme products, the same HPLC equipment with a semipreparative MultoHigh 100 Si column (250× 10 mm, 5 μm) at a flow rate of 2.5 mL min^{-1} was used. Conversion yields of enzyme products were calculated from peak areas in HPLC chromatograms.

NMR and mass spectrometric analyses

For structural elucidation, the isolated enzyme products were subjected to ^1H NMR and MS analyses. High-resolution electron impact (HR-EI) MS data were obtained on a Micromass Auto Spec spectrometer (Waters, Milford, MA) and are given in Table 2. For NMR analysis, the enzyme products were dissolved in CD_3OD (650 μL). Spectra were recorded at room temperature with an ECX-500 spectrometer (JEOL, Tokyo, Japan) equipped with a broadband probe with *z*-gradient. Chemical shifts were referenced to the solvent signal at 3.30 ppm. All spectra were processed with MestReNova 5.2.2 (Mestrelab Research, Santiago de Compostella, Spain). NMR data for the isolated products are given in Table 3 and spectra in Figs. S1–S9 in electronic supplementary data.

Results

Sequence analysis of the putative prenyltransferase gene AO090102000322

The putative prenyltransferase gene AO090102000322 is located on supercontig 13 of *A. oryzae* RIB40 at bp 868715-870055 (Aspergillus Comparative) (Galagan et al. 2005; Machida et al. 2005) and consists of only one exon of 1,341 bp. The deduced protein BAE61387 comprises 446 amino acids with a calculated molecular mass of 50.5 kDa. BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed low sequence similarity of BAE61387 with known proteins including prenyltransferases of the DMATS superfamily. For example, BAE61387 shares a sequence identity of 28 % with VrtC, a geranyltransferase mentioned above (Chooi et al. 2012), as well as with NscD, a dimethylallyltransferase from *N. fischeri* (Chooi et al. 2013). Both VrtC and NscD use polycyclic polyketide derivatives as aromatic substrates (Chooi et al. 2012, 2013). An orthologous gene AFLA_004300 (=AFL2G_09741, coding for EED47789) was found in the genome of the close relative *Aspergillus flavus* NRRL3357. Both orthologous genes have the same length of nucleotides and amino acid chains and share a sequence identity of 99 % on the amino acid level with each other. Analysis of the neighbouring genes of AO090102000322 on supercontig 13 did not indicate the presence of a putative gene cluster for secondary metabolites (Table S1 in electronic supplementary data). No genes encoding for non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS) or other known secondary metabolite backbone genes could be identified in the vicinity of the prenyltransferase gene AO090102000322. Similar situation was also found for AFLA_004300 in *A. flavus* NRRL3357. The natural substrate of BAE61387 and EED47789 can therefore not be predicted by sequence analysis.

Gene amplification and cloning, overproduction and purification of BAE61387-His_6

To prove the function of BAE61387, the coding region was amplified from genomic DNA of *A. oryzae* DSM1147 by PCR (see "Materials and methods"). The obtained PCR fragment of 1,351 bp was cloned into pGEM-T Easy and verified by sequencing. The coding region was then subcloned into the expression vector pQE60 at the *Nco*I and *Bgl*II sites to create the expression construct pDP004.

For gene expression, *E. coli* cells harbouring pDP004 were cultivated at 37 °C and induced for 16 h with isopropyl β-D-1-thiogalactopyranoside at a final concentration of 1.0 mM. The recombinant protein was purified on Ni-NTA-agarose resin, and a major protein band migrating above the 45 kDa marker band was detected on SDS-PAGE (Fig. 1). The protein size

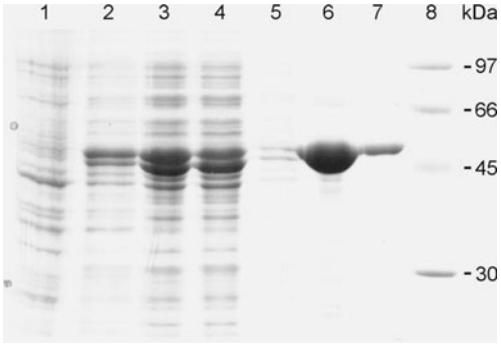


Fig. 1 SDS-PAGE analysis of the overproduction and purification of BAE61387-His_6. The proteins were separated on a 12 % SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. *Lane 1*: total protein before induction, *lane 2*: total protein after induction with IPTG, *lane 3*: soluble fraction after IPTG induction, *lane 4*: flow-through, *lane 5*: wash fraction, *lane 6*: first elution fraction, *lane 7*: second elution fraction, *lane 8*: molecular mass standard

corresponds well with the calculated molecular mass of BAE61387-His_6 at 51.5 kDa. A protein yield of 9.0 mg purified BAE61387-His_6 was obtained per litre culture. The molecular mass of the native recombinant BAE61387-His_6 was determined by size exclusion chromatography as 93.5 kDa, indicating that it presumably acts as a homodimer.

Enzyme activity and substrate specificity of BAE61387

BAE61387 was assayed in the presence of DMAPP with 34 aromatic substrates, which had been accepted by prenyltransferases of the DMATS superfamily as substrates. They included aromatic amino acids such as tryptophan and tyrosine (Yu et al. 2012; Zou et al. 2011), tryptophan-containing cyclic dipeptides (Yu and Li 2012), hydroxynaphthalenes (Yu et al. 2011), flavonoids (Yu and Li 2011) and xanthones (Pockrandt et al. 2012). The incubation mixtures were analysed on HPLC under routine conditions used in our group for detection of enzyme products. Clear product formation was detected in the incubation mixtures with 7 of 10 tested hydroxynaphthalenes. Low or no product formation was observed in the incubation mixtures with other tested substances (Table 1 and Table S2 in electronic supplementary materials). Assays of hydroxynaphthalenes with GPP and FPP led to detectable product formation in seven and five reaction mixtures, respectively. High conversion yields were achieved for 1-naphthol (**1a**), 1,7-dihydroxynaphthalene (**2a**) and 2,7-dihydroxynaphthalene (**3a**), which were also well accepted by some prenyltransferases of the DMATS superfamily in the presence of DMAPP (Yu et al. 2011). Therefore, the three substrates were chosen for further study. As shown in Fig. 2, one additional dominant peak each was detected in their enzyme assays with DMAPP, GPP and FPP. Product formation in all of the enzyme assays was strictly dependent on the presence of active enzyme and prenyl donors. Negative controls with heat-inactivated enzyme (boiled for 30 min) or without prenyl donor showed no additional peaks in HPLC chromatograms (data not shown). With an exception for the incubation mixture of **3a** and GPP, the enzymatic activity decreased with the increased chain length of the prenyl donor. For example, the conversion yields of the incubation mixtures of **1a-3a** with DMAPP were found between 20 and 72 %, followed by the incubation mixtures with GPP at 15–46 % and those with FPP at 5–18 %. In the incubation mixture of **3a** with GPP, a slightly higher conversion of 29 % was observed than in the enzyme assay with DMAPP of 20 %.

Table 1 Acceptance of hydroxynaphthalenes by BAE61387

Substrate	Conversion yield (%)		
	DMAPP	GPP	FPP
1-naphthol (**1a**)	51	15	5
2-naphthol	3	1	<0.04
1,4-dihydroxynaphthalene	<0.02	<0.02	<0.01
1,5-dihydroxynaphthalene	<0.04	<0.04	<0.04
1,6-dihydroxynaphthalene	12	4	3
1,7-dihydroxynaphthalene (**2a**)	72	46	18
2,3-dihydroxynaphthalene	<0.07	<0.05	<0.04
2,6-dihydroxynaphthalene	10	3	2
2,7-dihydroxynaphthalene (**3a**)	20	29	10
8-amino-2-naphthoat	5	2	<0.03

Enzyme assays (100 μL) contained Tris-HCl buffer (50 mM, pH 7.5), aromatic substrate (1.0 mM), DMAPP, GPP or FPP (2 mM), BAE61387 (40 μg), $CaCl_2$ (5 mM), glycerol (3 %*v*/*v*) and dimethyl sulfoxide (DMSO; 2 %*v*/*v*). The reaction mixtures were incubated at 37 °C for 16 h

BAE61387 catalyses the same regioselective C-prenylation of hydroxynaphthalenes with DMAPP, GPP and FPP

For structural elucidation, the enzyme products **1b-1d**, **2b-2d** and **3b-3d** (Fig. 2) were isolated by preparative HPLC from the incubation mixtures of **1a, 2a** and **3a,** respectively, and subjected to ^{1}H NMR and MS analyses. High-resolution (HR) MS (Table 2) confirmed the presence of the respective prenyl moiety in the enzyme products. The molecular masses of **1b-3b** are 68 Da larger than those of **1a-3a**, respectively, proving the attachment of a dimethylallyl moiety. The geranylated **1c-3c** and farnesylated **1d-3d** are 136 and 204 Da larger than their respective substrates **1a-3a**.

^{1}H NMR spectra of enzyme products from the same aromatic substrate, i.e. **1b-1d**, **2b-2d** or **3b-3d** (Table 3 and Figs. S1–S9 in electronic supplementary materials), showed signals with same number and coupling pattern of aromatic protons to each other. This indicated that the prenylation position of the enzyme products of a given substrate remains the same, despite different prenyl donors (DMAPP, GPP or FPP). The chemical shifts of H-1′ of the prenyl moieties for all of the isolated enzyme products were found between 3.36–

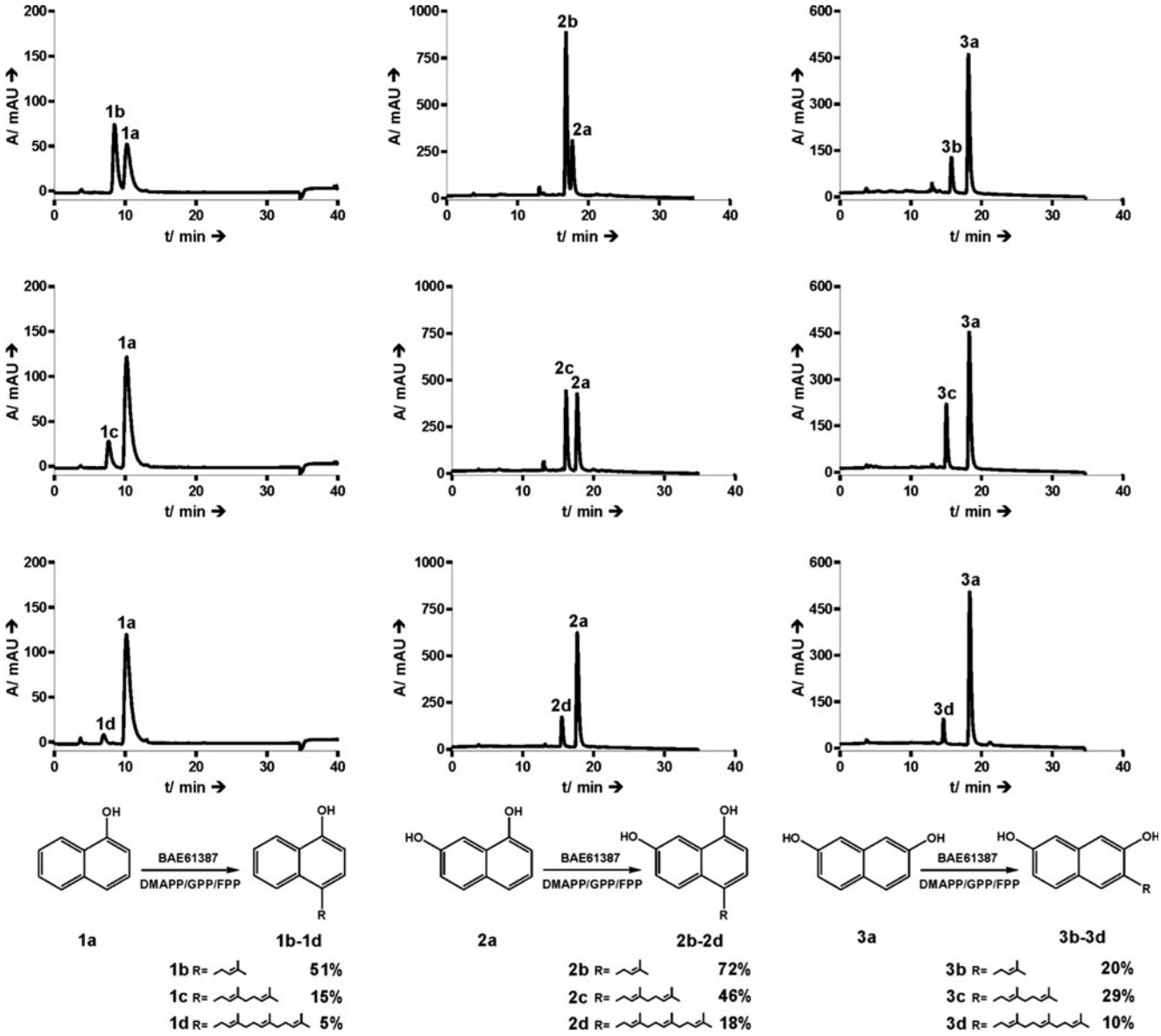


Fig. 2 HPLC analysis of the enzyme assays of BAE61387 with hydroxynaphthalenes on a MultoHigh 100 Si column. Standard reaction mixtures contained 40 μg of BAE61387, 1.0 mM of aromatic substrate, 2.0 mM DMAPP, GPP or FPP, 5 mM $CaCl_2$ and 50 mM Tris-HCl at pH 7.5 and were incubated at 37 °C for 16 h. The illustrated chromatograms were recorded at 254 nm. Conversion yields are given under the reaction scheme

3.63 ppm, proving their attachment to a C-atom rather than to an oxygen. In the ^{1}H NMR spectra of **1b-1d** and **2b-2d**, signals for two new coupling aromatic protons each with coupling constants of 7.6 or 7.7 Hz were detected, instead of three coupling aromatic protons with similar coupling constants in those of **1a** and **2a** (data not shown). This proved that **1a** and **2a** were prenylated at C-4, i.e. the *para*-position of a hydroxyl group of the same benzene ring, in the presence of DMAPP, GPP and FPP. In the ^{1}H NMR spectra of **3b-3d**, two singlets each were detected, instead of three coupling aromatic protons as an ABM system in **3a** (data not shown). This led to the conclusion that **3a** was prenylated at C-3, i.e. *ortho*-position of a hydroxyl group, in the presence of DMAPP, GPP and FPP (Fig. 2). Prenylations of hydroxynaphthalenes at *para*- or *ortho*-positions of a hydroxyl group have been observed for some prenyltransferases of the DMATS superfamily in the presence of DMAPP (Yu et al. 2011). The NMR data of **1b**, **2b** and **3b** corresponded well to those reported previously (Yu et al. 2011).

Biochemical properties and determination of the kinetic parameters of BAE61387 with DMAPP, GPP and FPP

To test the dependency of the BAE61387 reaction on metal ions, we assayed **2a** with DMAPP in the presence of diverse metal ions at final concentrations of 5 mM. Incubation

Table 2 HR-EI-MS data of the isolated enzyme products

Compd.	Chemical formula	HR-EI-MS data		Deviation (ppm)
		calculated	measured	
1b	$C_{15}H_{16}O$	212.1201	212.1208	3.3
1c	$C_{20}H_{24}O$	280.1827	280.1840	4.6
1d	$C_{25}H_{32}O$	348.2453	348.2446	−2.0
2b	$C_{15}H_{16}O_2$	228.1150	228.1131	−8.3
2c	$C_{20}H_{24}O_2$	296.1776	296.1792	5.4
2d	$C_{25}H_{32}O_2$	364.2402	364.2394	−2.2
3b	$C_{15}H_{16}O_2$	228.1150	228.1154	1.8
3c	$C_{20}H_{24}O_2$	296.1776	296.1786	3.4
3d	$C_{25}H_{32}O_2$	364.2402	364.2398	−1.1

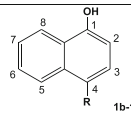
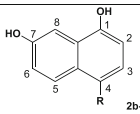
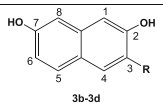
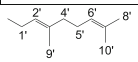
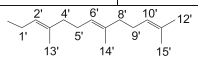
mixtures without additives or with EDTA were used as controls. Our results showed that BAE61387 catalyses its reaction also in the presence of the chelating agent EDTA. However, addition of Ca^{2+} increased the enzyme activity about 40 % (Fig. S10). These results corresponded well with those observed for other members of the DMATS superfamily (Li 2009; Yu and Li 2012).

Kinetic parameters including Michaelis-Menten constant (K_M) and turnover number (k_{cat}) were determined for **1a-3a** and DMAPP, GPP and FPP (Table 4 and Figs. S11–S22 in electronic supplementary data). The enzyme reaction of BAE61387 with hydroxynaphthalenes and DMAPP, GPP and FPP apparently followed Michaelis-Menten kinetics. For the tested three substrates, their affinities to the enzyme decreased with the increased chain length of prenyl donors. In the presence of DMAPP, **2a** was accepted as the best substrate with a K_M value at 0.56 mM, followed by **1a** at 1.01 mM and **3a** at 1.85 mM. These values are somewhat higher than those observed for other prenyltransferases with their natural substrates (Yu and Li 2012). In the presence of GPP, K_M values for **1a**, **2a** and **3a** were determined at 1.55, 1.42 and 1.94 mM, respectively. In the presence of FPP, K_M values for **1a**, **2a** and **3a** were found to be 2.08, 1.73 and 2.81 mM. The affinity of different prenyl donors to the enzyme was found to be comparable. In the presence of **2a**, FPP with a K_M value of 1.03 mM showed only slightly higher affinity than DMAPP with a K_M value of 1.28 mM. The velocity of the BAE61387 reaction in the presence of hydroxynaphthalenes was relative low. Turnover numbers of approximately 0.03 s^{-1} were calculated for **1a-3a** in the presence of the best accepted prenyl donor DMAPP, indicating these substances are not its natural substrates.

Discussion

In this study, we cloned and expressed a putative prenyltransferase gene AO090102000322 from *A. oryzae*, which shares meaningful sequence similarity with known members of the DMATS superfamily (Yu and Li 2012). Incubation of the recombinant enzyme BAE61387 with diverse aromatic substrates showed its ability to prenylate hydroxynaphthalenes in the presence of different prenyl donors such as DMAPP, GPP, or FPP.

Table 3 ^{1}H-NMR data of the enzyme products in CD_3OD

Compd.	 1b-1d	 2b-2d	 3b-3d						
	DMA: 	Geranyl: 	Farnesyl: 						
pos.	**1b R=DMA**	**1c R=Geranyl**	**1d R=Farnesyl**	**2b R=DMA**	**2c R=Geranyl**	**2d R=Farnesyl**	**3b R=DMA**	**3c R=Geranyl**	**3d R=Farnesyl**
	-	-	-	-	-	-	6.85, s	6.85, s	6.85, s
2	6.69, d, 7.7	6.70, d, 7.7	6.69, d, 7.7	6.62, d, 7.6	6.62, d, 7.6	6.61, d, 7.6	-	-	-
3	7.07, d, 7.7	7.08, d, 7.7	7.08, d, 7.7	6.85, d, 7.6	6.87, d, 7.6	6.87, d, 7.6	-	-	-
4	-	-	-	-	-	-	7.33, s	7.34, s	7.34, s
5	7.88, d, 7.5	7.88, d, 7.7	7.88, d, 8.2	7.75, d, 9.1	7.75, d, 9.1	7.75, d, 9.1	7.47, d, 8.6	7.46, d, 8.7	7.45, d, 8.7
6	7.44, ddd, 8.4, 6.8, 1.5	7.44, ddd, 8.4, 6.8, 1.5	7.43, ddd, 8.3, 6.9, 1.4	7.02, dd, 9.1, 2.6	7.02, dd, 9.1, 2.6	7.02, dd, 9.1, 2.6	6.77, dd, 8.7, 2.4	6.77, dd, 8.7, 2.4	6.77, dd, 8.7, 2.4
7	7.38, ddd, 8.1, 6.8, 1.2	7.38, ddd, 8.1, 6.8, 1.2	7.38, ddd, 8.0, 6.9, 1.1	-	-	-	-	-	-
8	8.19, dd, 8.3, 0.9	8.19, dd, 8.3, 0.9	8.19, dd, 8.4, 0.9	7.47, d, 2.6	7.47, d, 2.6	7.47, d, 2.6	6.83, d, 2.3	6.83, d, 2.4	6.83, d, 2.5
1'	3.63, d, 7.0	3.64, d, 7.0	3.64, d, 6.8	3.58, d, 7.0	3.58, d, 7.0	3.59, d, 6.9	3.36, d, 7.2	3.37, d, 7.2	3.37, d, 7.3
2'	5.31, br. t, 7.0	5.32, br. t, 7.0	5.32, t, 6.8	5.29, t, 7.0	5.29, t, 7.0	5.30, t, 6.9	5.39, t, 7.2	5.40, t, 7.2	5.40, t, 7.3
4'	1.78, s	2.10, m	2.11, m	1.77, s	2.09, m	2.10, m	1.75, s	2.12, m	2.14, m
5'	1.72, s	2.04, m	2.05, m	1.71, s	2.03, m	2.04, m	1.72, s	2.06, m	2.08, m
6'	-	5.06, br. t, 6.9	5.07, br. t, 6.9	-	5.06, t, 6.8	5.08, t, 6.8	-	5.12, t, 7.6	5.14, t, 6.9
8'	-	1.77, s	1.98, m	-	1.75, s	1.98, m	-	1.71, s	2.00, m
9'	-	1.60, s	1.89, m	-	1.60, s	1.90, m	-	1.64, s	1.93, m
10'	-	1.55, s	5.03, br. t, 7.1	-	1.55, s	5.03, t, 6.9	-	1.58, s	5.04, t, 7.1
12'	-	-	1.54, s	-	-	1.55, s	-	-	1.53, s
13'	-	-	1.78, s	-	-	1.76, s	-	-	1.71, s
14'	-	-	1.62, s	-	-	1.62, s	-	-	1.62, s
15'	-	-	1.54, s	-	-	1.55, s	-	-	1.58, s

Chemical shifts are given in parts per million and coupling constants in hertz

Table 4 Kinetic parameters of BAE61387

Compd.	K_M [mM]			k_{cat} [s^{-1}]			k_{cat}/K_M [$s^{-1}M^{-1}$]		
	DMAPP	GPP	FPP	DMAPP	GPP	FPP	DMAPP	GPP	FPP
1a	1.01±0.15	1.55±0.17	2.08±0.38	0.03±0.005	0.01±0.001	0.006±0.0003	30	6.5	3
2a	0.56±0.06	1.42±0.12	1.73±0.20	0.03±0.001	0.03±0.003	0.007±0.001	54	21	4
3a	1.85±0.15	1.94±0.11	2.81±0.14	0.03±0.003	0.02±0.001	0.005±0.0003	16	10	2
DMAPP[a]	1.28±0.30	–	–	0.025±0.004	–	–	20	–	–
GPP[a]	–	1.77±0.08	–	–	0.022±0.0001	–	–	12	–
FPP[a]	–	–	1.03±0.06	–	–	0.01±0.0004	–	–	10

[a] Kinetic parameters were measured with **2a** as aromatic substrate

Literature search did not reveal any report on prenylated hydroxynaphthalenes or derivatives thereof from *A. oryzae*. Analysis of the genes up- and downstream of AO090102000322 indicate the presence of neither polyketide synthase genes for the formation of a naphthalene backbone (Chooi and Tang 2012; Fujii et al. 2000) nor non-ribosomal peptide synthetase for a peptide backbone (Condurso and Bruner 2012) or other biosynthetic genes of secondary metabolites (Table S1). Prenylated hydroxynaphthalenes are a group of secondary metabolites mainly isolated from plants (Hussein et al. 2003, 2004) and bacteria (Kumano et al. 2010; Shin-Ya et al. 1990). In bacteria, prenyltransferases of the CloQ/NphB group are responsible for the prenylation of hydroxynaphthalenes. Examples are NphB (also called Orf2) in the biosynthesis of naphterpin in *Streptomyces* sp. strain CL190 (Kumano et al. 2008; Kuzuyama et al. 2005), Fnq26 in the biosynthesis of furanonaphthoquinone I in *Streptomyces cinnamonensis* DSM 1042 (Haagen et al. 2007) and Fur7 in the biosynthesis of furaquinocin in *Streptomyces* sp. strain KO-3988 (Kumano et al. 2010). All the mentioned enzymes used GPP as prenyl donor. Several homologous genes from different fungi like *Aspergillus terreus*, *Botryotinia fuckeliana* and *Sclerotinia sclerotiorum* were found to catalyse the prenylation of 2,7-dihydroxynaphthalene. These fungal enzymes share conserved amino acid residues of the members of CloQ/NphB group and used DMAPP as prenyl donor (Haug-Schifferdecker et al. 2010). BAE61387 shares sequence similarity with hydroxynaphthalene prenyltransferases from neither bacteria nor fungi. Therefore, a hydroxynaphthalene is unlikely the natural substrate of BAE61387. The acceptance of hydroxynaphthalenes by BAE61387 demonstrated just its high flexibility towards aromatic substrates, as observed for several members of the DMATS superfamily (Yu et al. 2011). The low affinity and turnover numbers of BAE61387 for hydroxynaphthalenes (Table 4) provided strong supports for this hypothesis. The natural substrate of BAE61387 remains unknown.

Remarkably, BAE61387 used DMAPP, GPP and FPP as prenyl donors, which had not been reported for an aromatic prenyltransferase prior to this study. The promiscuous bacterial geranyltransferase Fur7 used also DMAPP as prenyl donor for its prenylation of hydroxynaphthalenes (Kumano et al. 2010), and the fungal indole dimethylallyltransferase AnaPT accepted both DMAPP and GPP as prenyl donors (Pockrandt and Li 2013). However, there are no reports on an acceptance of FPP by these enzymes. Acceptance of DMAPP, GPP and FPP by BAE61387 could indicate a flexible biding site for prenyl donors in the reaction chamber of this enzyme.

Attachment of C_5-, C_{10}- and C_{15}- units to naphthalene backbone increases the structural diversity of prenylated compounds, and regiospecific prenylation would be welcome for structure modification. BAE61387 catalysed the monoprenylation of hydroxynaphthalenes at *para*- or *ortho*-position of a hydroxyl group, but not at both positions. Therefore, one predominant product each was obtained from incubation mixtures of **1a**-**3a** with DMAPP, GPP and FPP. In comparison, products with geranylations at *para*- and *ortho*-positions have been isolated from incubation mixtures of NphB with a given hydroxynaphthalene (Kumano et al. 2008).

We have recently demonstrated that the prenylation positions of indole derivatives were often shifted to other positions of the indole ring by dimethylallyltransferases of the DMATS superfamily, when DMAPP analogues, GPP or benzyl diphosphate instead of DMAPP itself were used as alkyl donors (Liebhold et al. 2012, 2013; Liebhold and Li 2013; Pockrandt and Li 2013). BAE61387 catalysed the prenyl transfer reactions to the same position, i.e. *para*- or *ortho*-position to a hydroxyl group despite different chain length of the prenyl donors. It seems that one of the activated positions at the naphthalene ring is the most preferable prenylation position of BAE61387 reaction.

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Electronic supplementary material for:

A Promiscuous Prenyltransferase from *Aspergillus oryzae* catalyses C-prenylations of hydroxynaphthalenes in the presence of different prenyl donors

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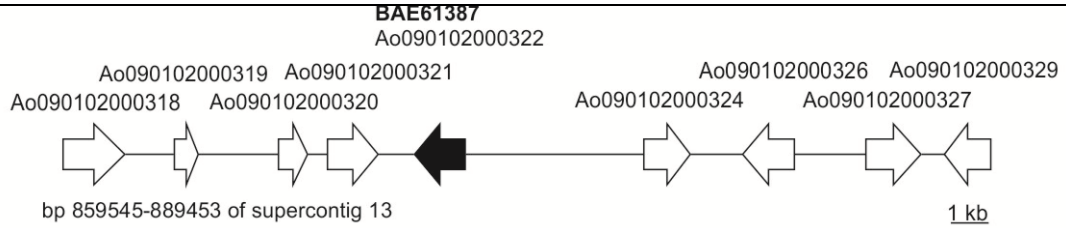
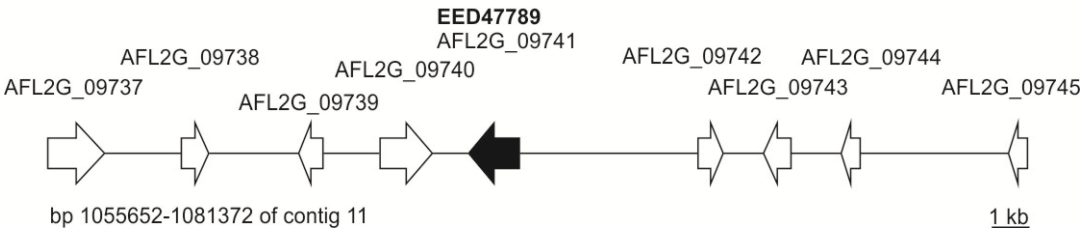
Table S1: Sequence analysis of neighboring genes of AO090102000322 and AFL2G_09741 (Aspergillus Comparative).



Gene	Putative function (NCBI conserved domain search)
AO090102000318	Phosphotransferase enzyme family protein
AO090102000319	No conserved domains
AO090102000320	No conserved domains
AO090102000321	Aminoglycoside 3'-phosphotransferase (APH) and Choline Kinase (ChoK) family protein
AO090102000322	DMATS prenyltransferase
AO090102000324	Ankyrin repeat domains
AO090102000326	No conserved domains
AO090102000327	Phosphatidylserine decarboxylase superfamily protein
AO090102000329	Alcohol dehydrogenase-like family protein



AFL2G_09737	Beta lactamase superfamily protein
AFL2G_09738	No conserved domains
AFL2G_09739	No conserved domains
AFL2G_09740	Aminoglycoside 3'-phosphotransferase (APH) and Choline Kinase (ChoK) family protein
AFL2G_09741	DMATS prenyltransferase
AFL2G_09742	No conserved domains
AFL2G_09743	No conserved domains
AFL2G_09744	No conserved domains
AFL2G_09745	No conserved domains

Table S2: Additional substrates tested in this study and conversion yields.

compound	Conversion yield		
	DMAPP	**GPP**	**FPP**
amino acids			
L-Trp	< 0.05 %	n. d.	n. d.
L-abrine	< 0.03%	n. d.	n. d.
L-Tyr	< 0.1 %	n. d.	n. d.
D-Tyr	< 0.1 %	n. d.	n. d.
tryptophan-containing cyclic dipeptides			
cyclo-L-Trp-L-Leu	4%	< 0.03	< 0.03.
cyclo-L-Trp-L-Tyr	< 0.02 %	n. d.	n. d.
cyclo-L-Trp-L-Trp	< 0.01 %	n. d.	n. d.
cyclo-L-Trp-L-Phe	< 0.05 %	n. d.	n. d.
cyclo-L-Trp-L-Ala	< 0.03 %	n. d.	n. d.
cyclo-D-Trp-L-Ala	< 0.03 %	n. d.	n. d.
(*R*)-benzodiazepinedione	< 0.01 %	n. d.	n. d.
(*S*)-benzodiazepinedione	< 0.02 %	n. d.	n. d.
brevianamide F	< 0.05 %	n. d.	n. d.
xanthone derivatives			
1,3,6-trihydroxyxanthone	< 0.01%	n. d.	n. d.
1,3,7-trihydroxyxanthone	< 0.03%	n. d.	n. d.
1,3,6,8-tetrahydroxyxanthone	< 0.02%	n. d.	n. d.
1,7-dihydroxy-6-methylxanthone	< 0.03%	n. d.	n. d.
1,7-dihydroxy-6,8-dimethylxanthone	< 0.03%	n. d.	n. d.
1,7-dihydroxy-5,6,8-trimethylxanthon	< 0.02%	n. d.	n. d.
flavonoids			
apigenin	< 0.1%	n. d.	n. d.
fisetin	< 0.2%	n. d.	n. d.
delphinidinchloride	< 0.1%	n. d.	n. d.
genistein	< 0.03%	n. d.	n. d.
naringenin	< 0.01%	n. d.	n. d.

n. d. = not determined

Enzyme assays (100 µL) contained Tris-HCl buffer (50 mM, pH 7.5), aromatic substrate (1.0 mM), DMAPP, GPP or FPP (2 mM), BAE61387 (40 µg), $CaCl_2$ (5 mM), glycerol (3% v/v) and dimethyl sulfoxide (DMSO; 2% v/v). The reaction mixtures were incubated at 37 °C for 16 h.

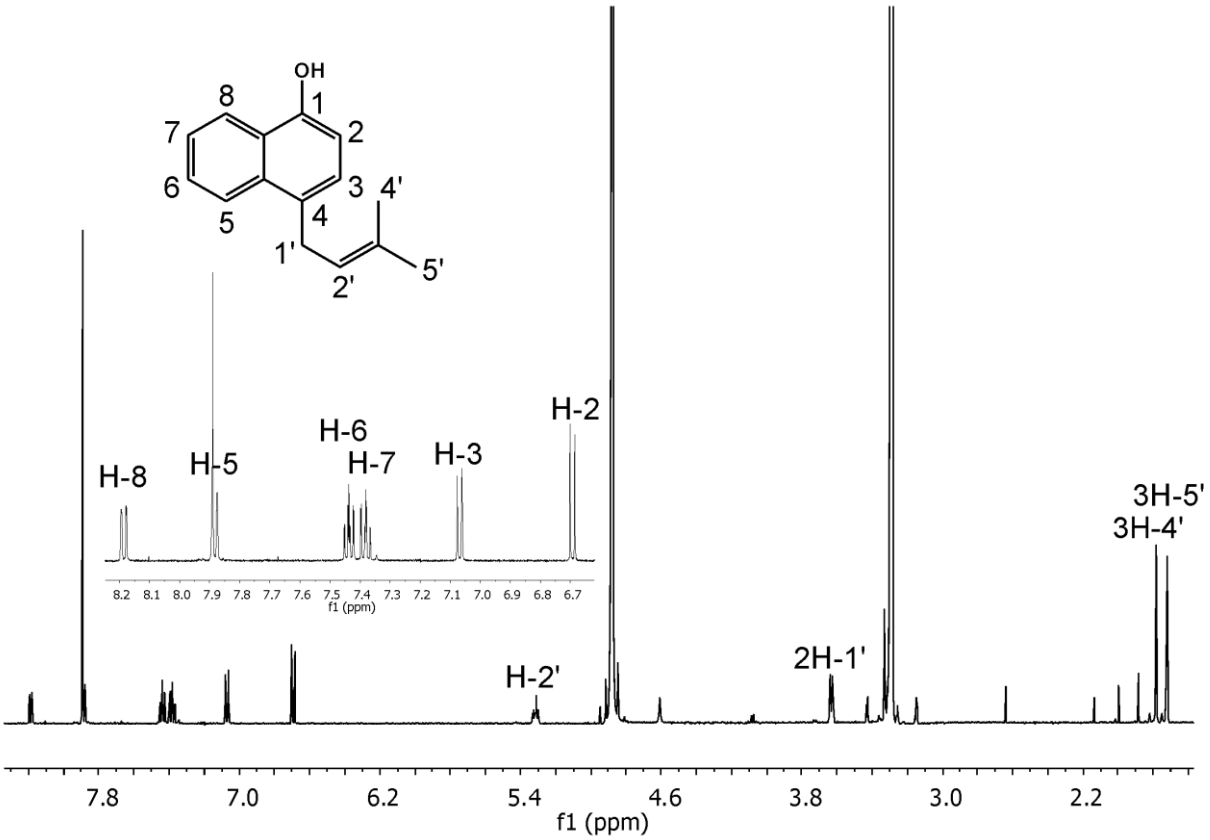


Figure S1: ^{1}H-NMR spectrum of **1b** in MeOH-d_4.

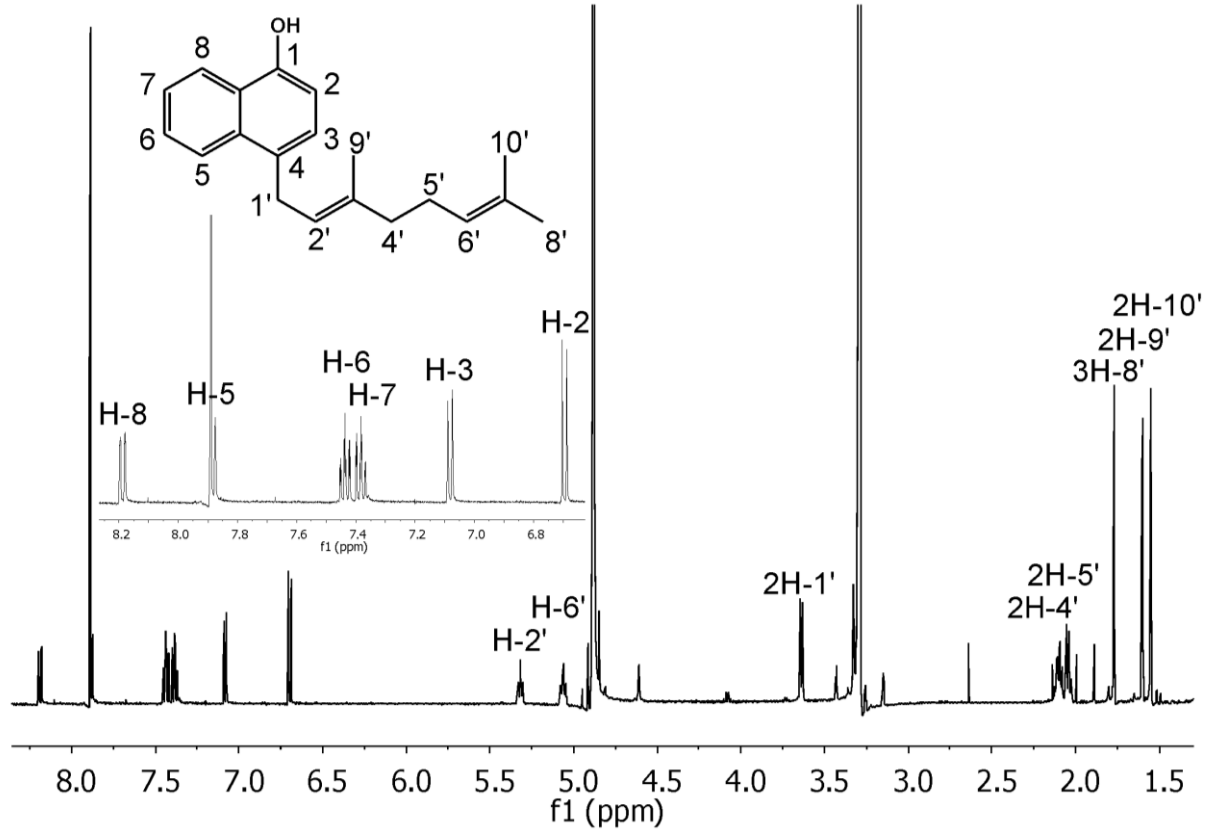


Figure S2: ^{1}H-NMR spectrum of **1c** in MeOH-d_4.

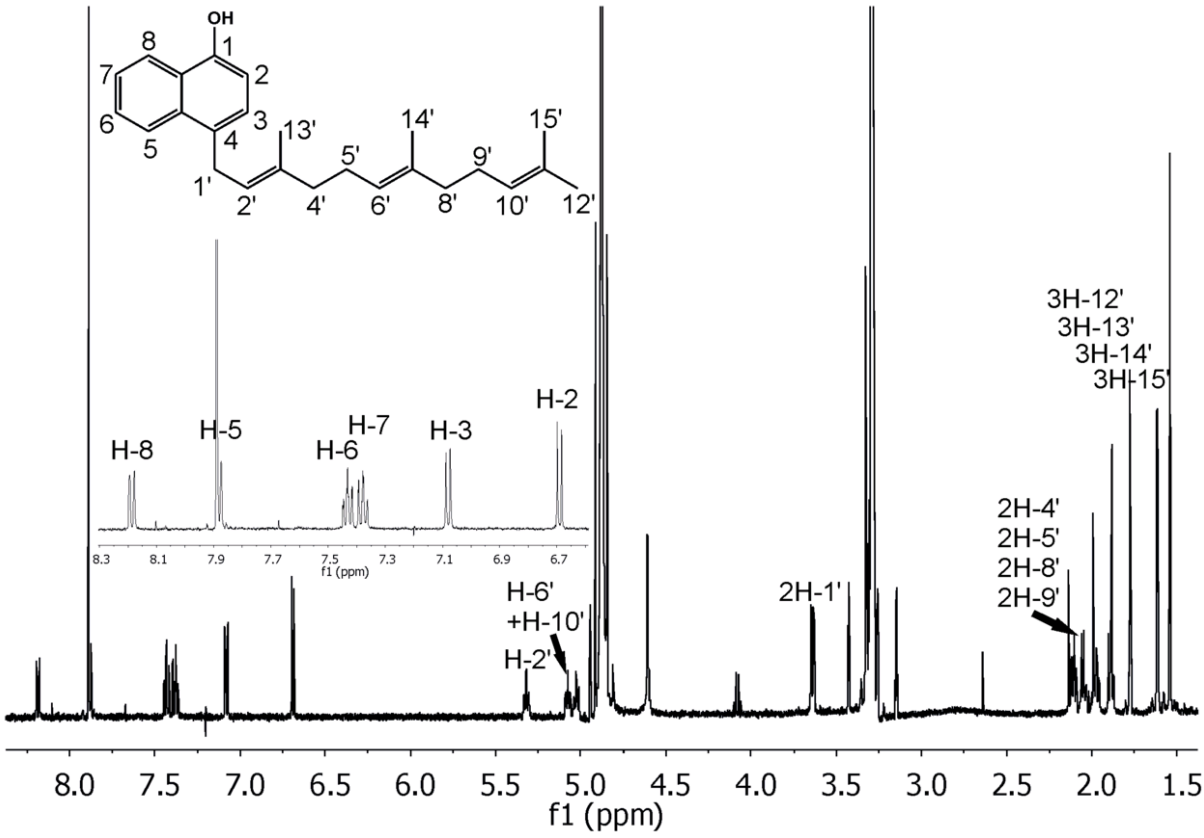


Figure S3: ^{1}H-NMR spectrum of **1d** in MeOH-d_4.

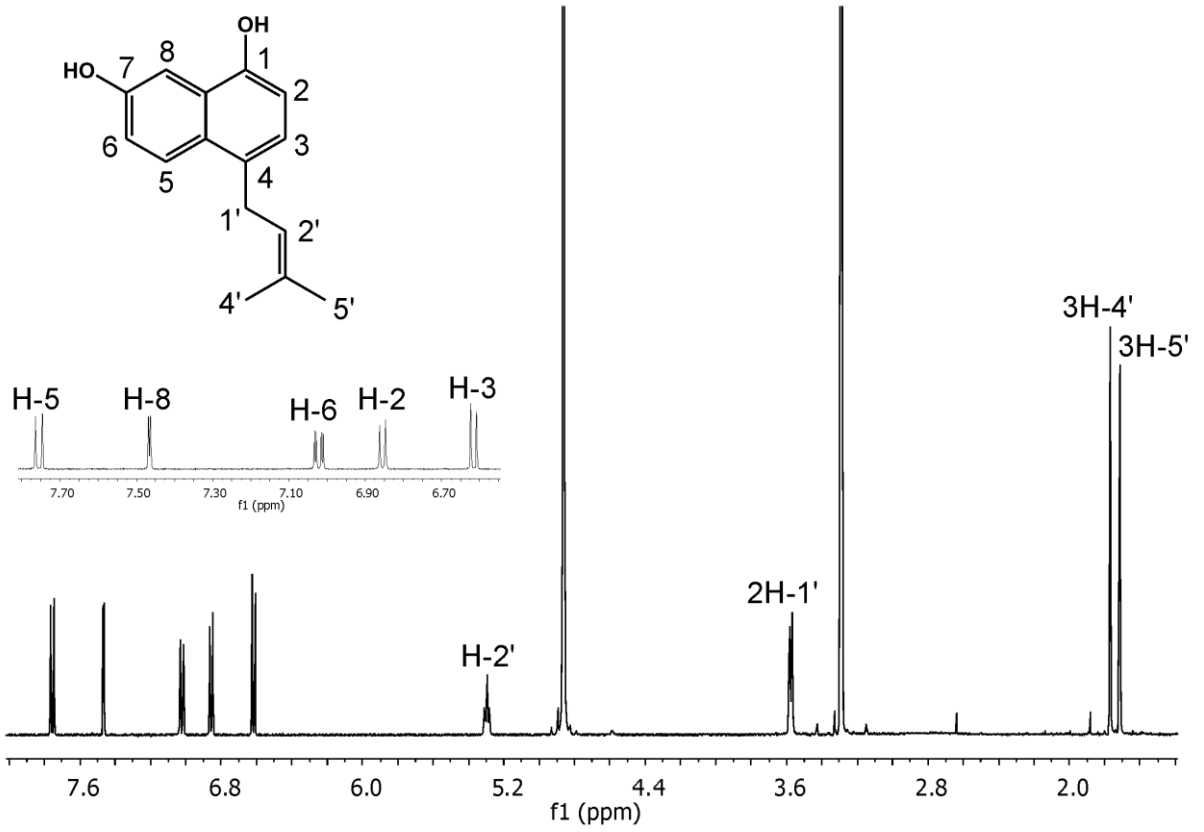


Figure S4: ^{1}H-NMR spectrum of **2b** in MeOH-d_4.

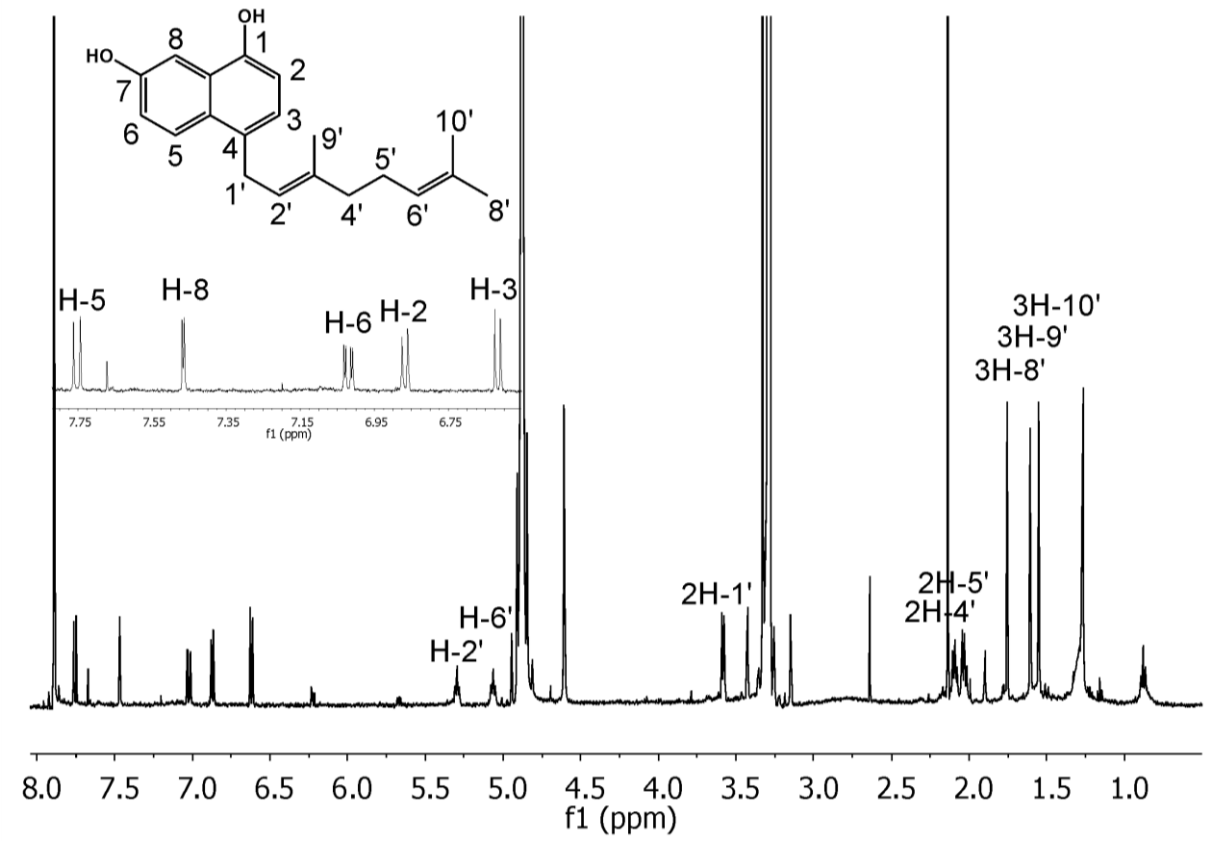


Figure S5: ^{1}H-NMR spectrum of **2c** in MeOH-d_4.

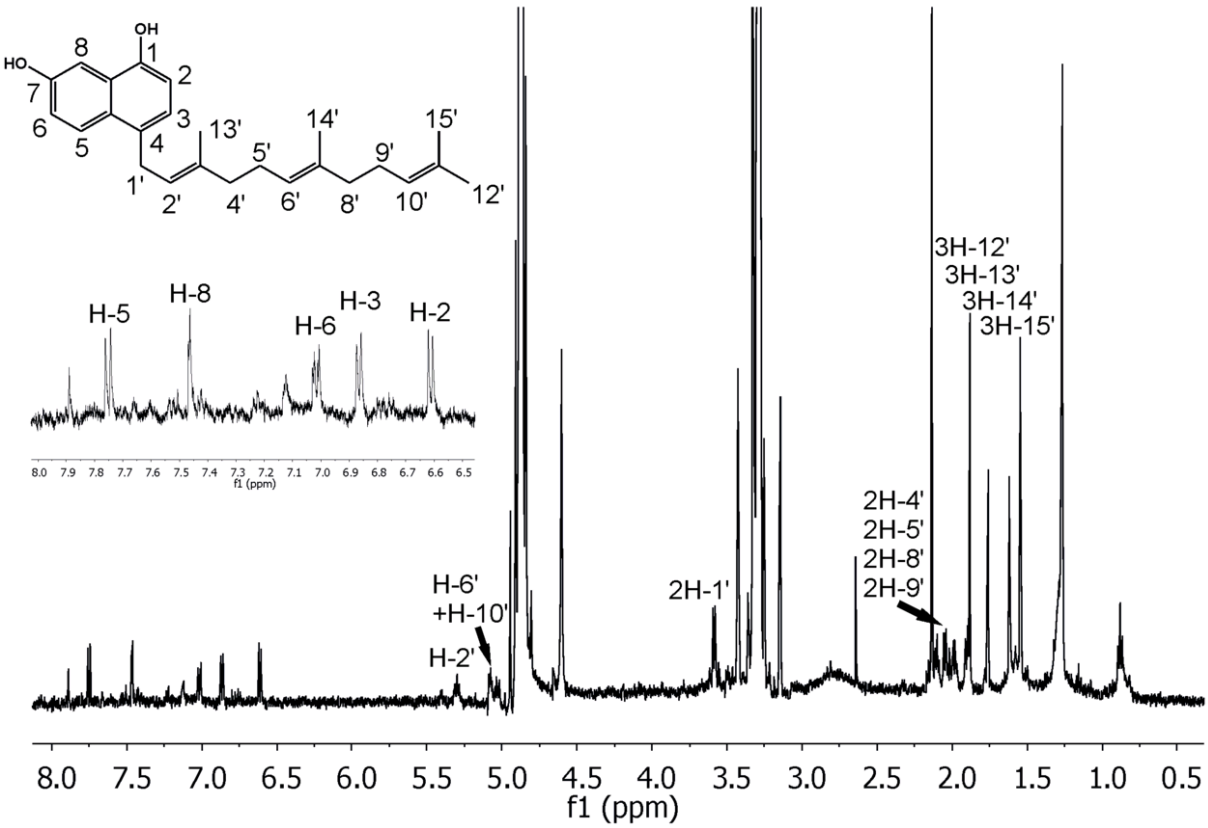


Figure S6: ^{1}H-NMR spectrum of **2d** in MeOH-d_4.

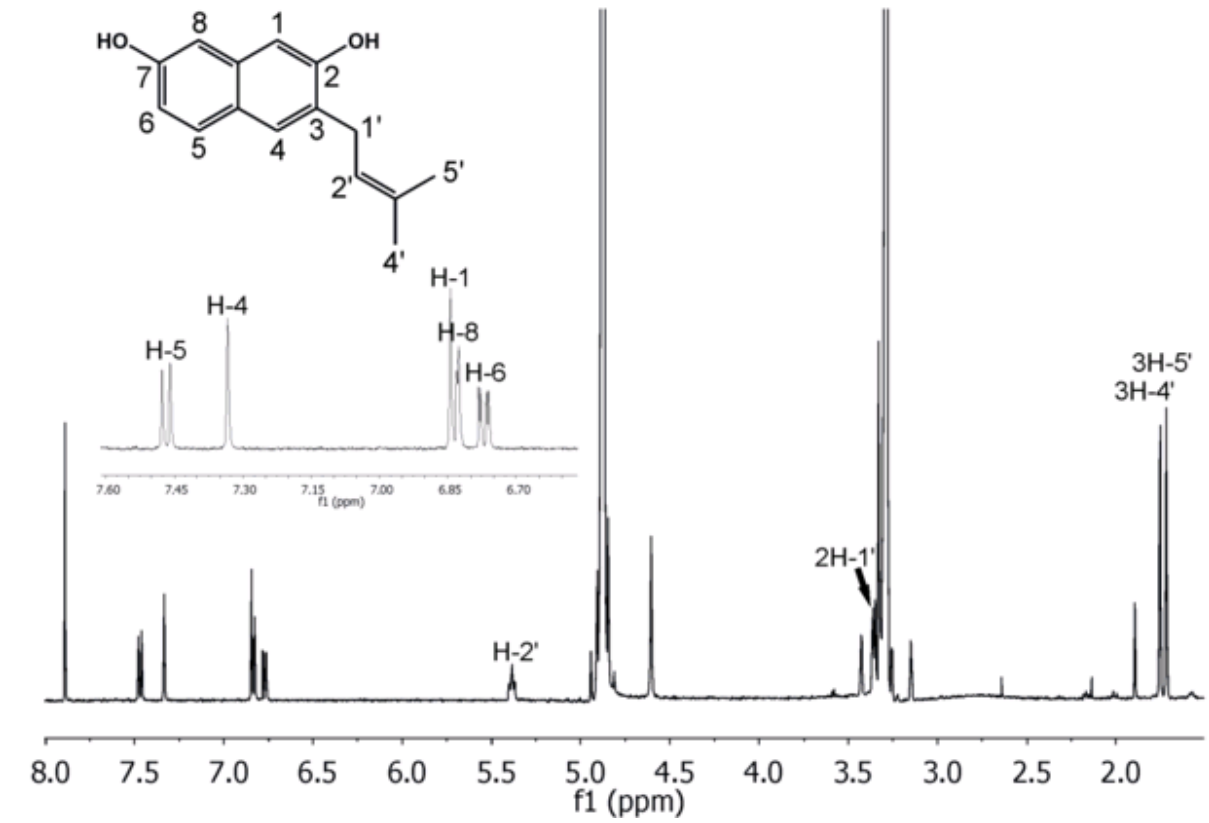


Figure S7: ^{1}H-NMR spectrum of **3b** in MeOH-d_4.

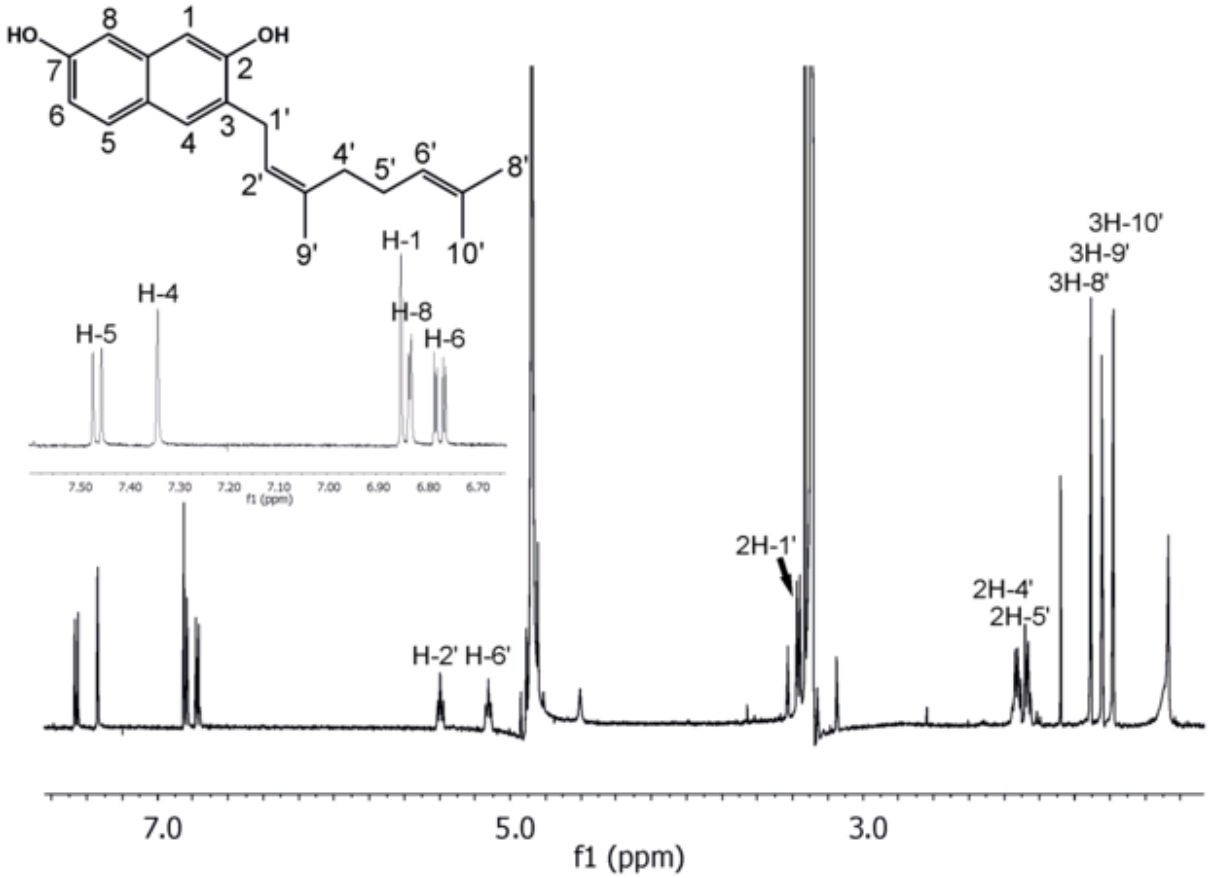


Figure S8: ^{1}H-NMR spectrum of **3c** in MeOH-d_4.

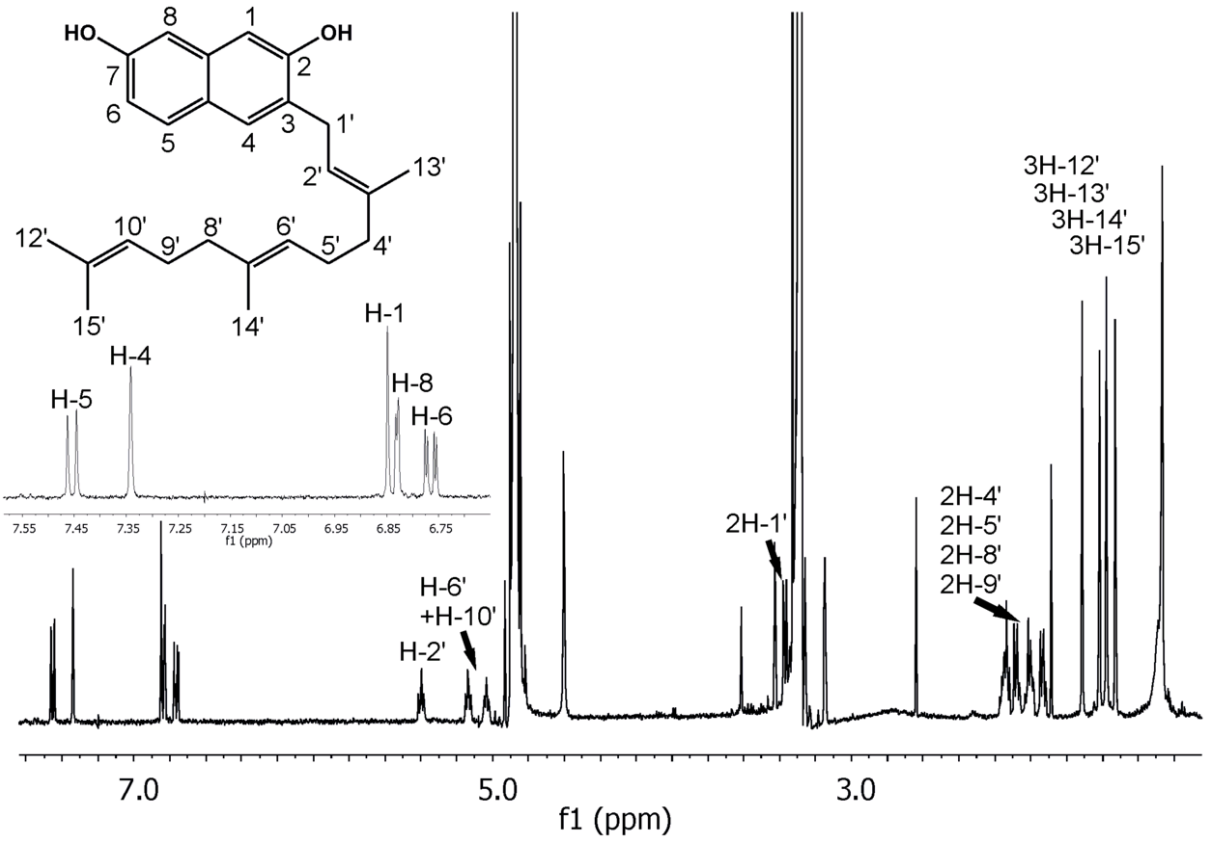


Figure S9: ^{1}H-NMR spectrum of **3d** in MeOH-d_4.

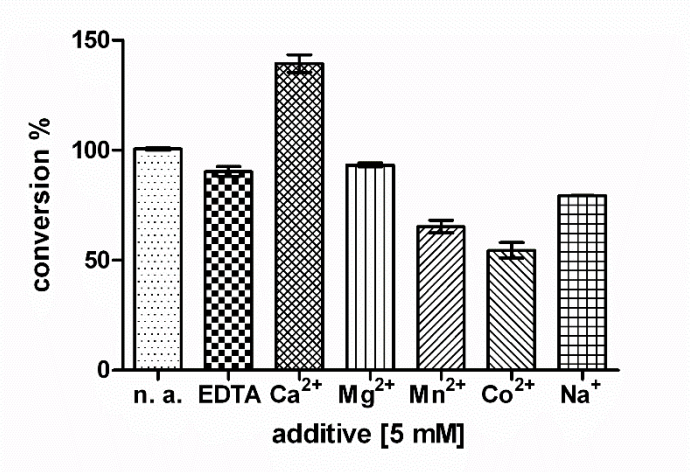


Figure S10: Ion dependency of BAE61387 with DMAPP using 1,7-dihydroxynaphthalene (**2a**) as aromatic substrate.

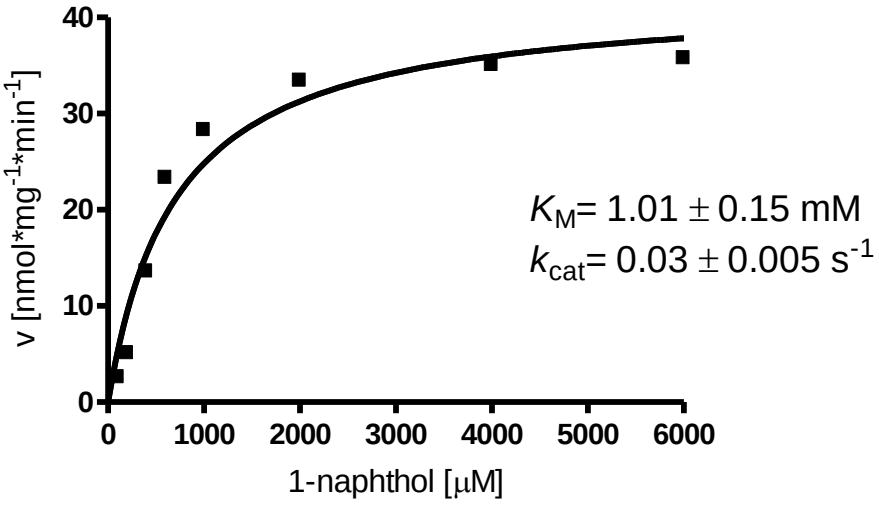


Figure S11: Determination of kinetic parameters for BAE61387 with 1-naphthol (**1a**) and DMAPP.

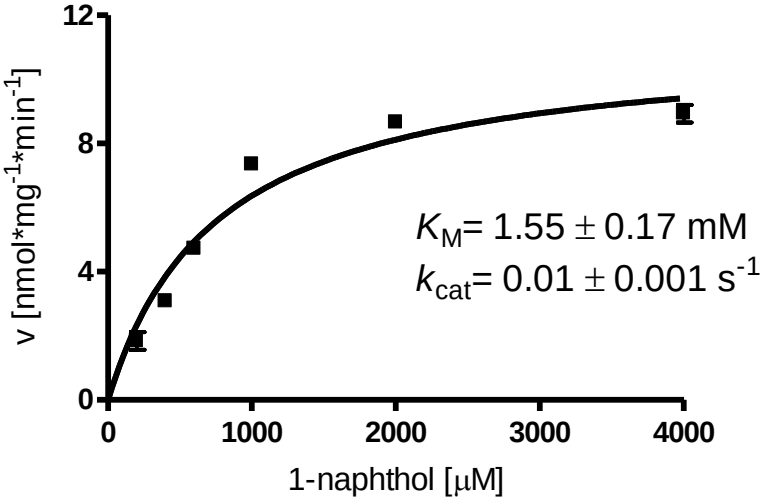


Figure S12: Determination of kinetic parameters for BAE61387 with 1-naphthol (**1a**) and GPP.

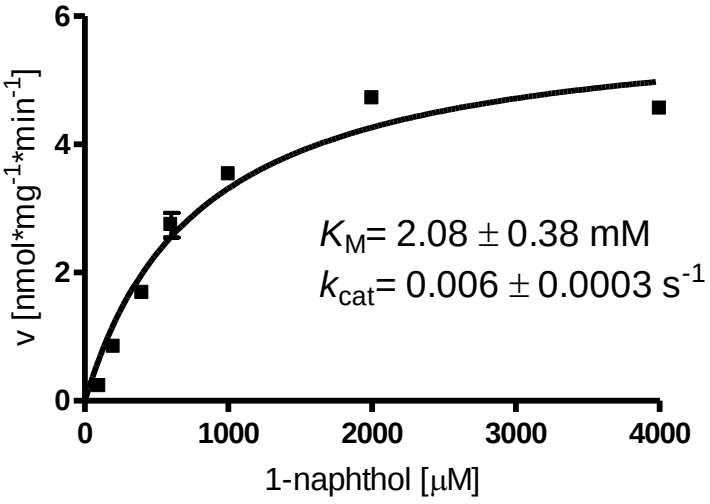


Figure S13: Determination of kinetic parameters for BAE61387 with 1-naphthol (**1a**) and FPP.

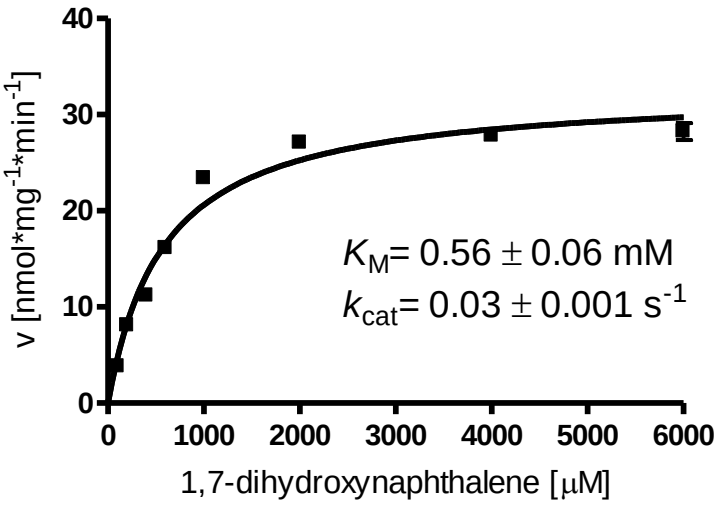


Figure S14: Determination of kinetic parameters for BAE61387 with 1,7-dihydroxynaphthalene (**2a**) and DMAPP.

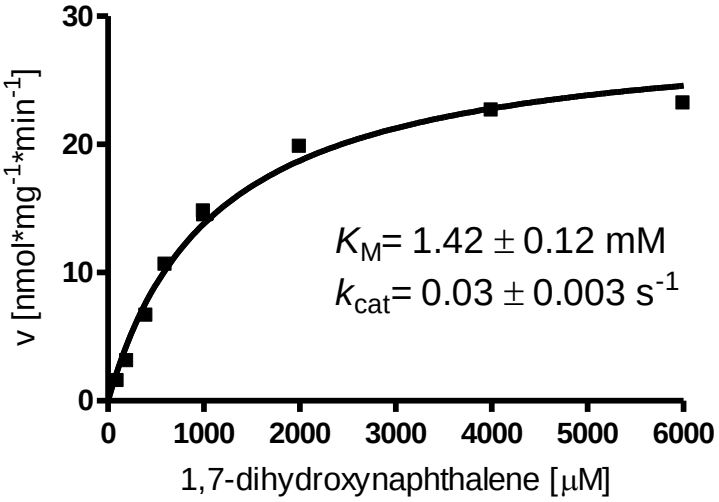


Figure S15: Determination of kinetic parameters for BAE61387 with 1,7-dihydroxynaphthalene (**2a**) and GPP.

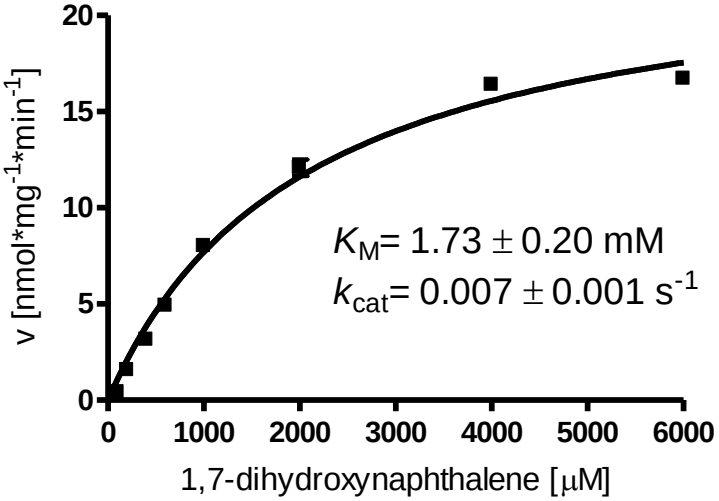


Figure S16: Determination of kinetic parameters for BAE61387 with 1,7-dihydroxynaphthalene (**2a**) and FPP.

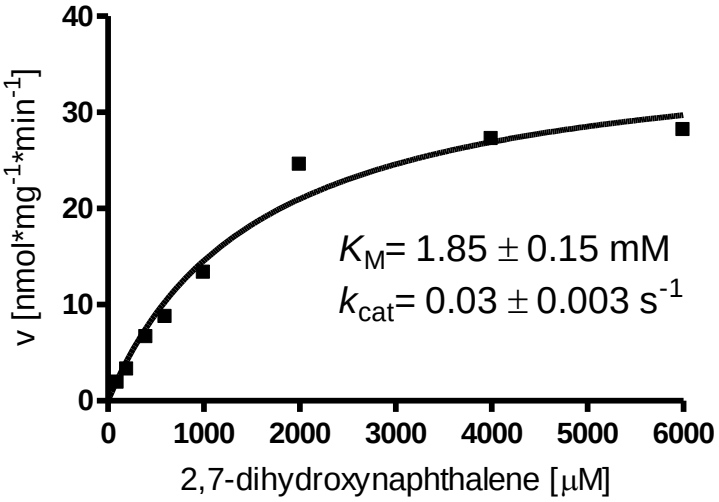


Figure S17: Determination of kinetic parameters for BAE61387 with 2,7-dihydroxynaphthalene (**3a**) and DMAPP.

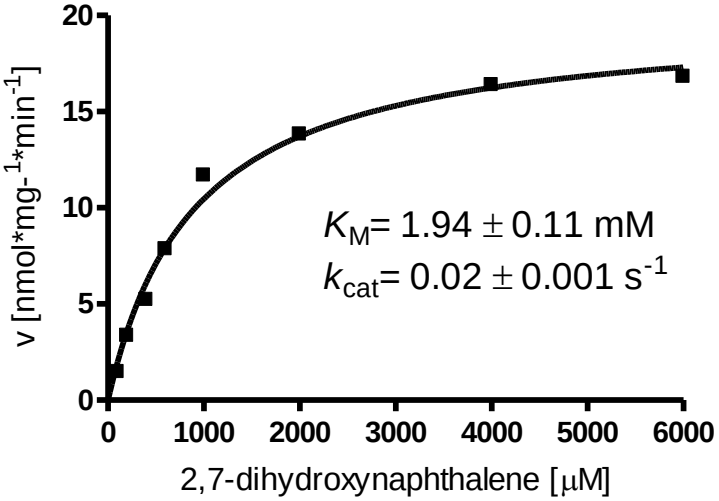


Figure S18: Determination of kinetic parameters for BAE61387 with 2,7-dihydroxynaphthalene (**3a**) and GPP.

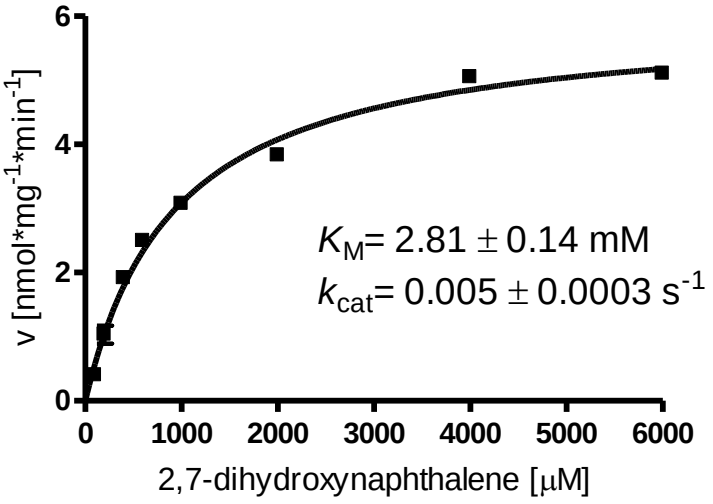


Figure S19: Determination of kinetic parameters for BAE61387 with 2,7-dihydroxynaphthalene (**3a**) and FPP.

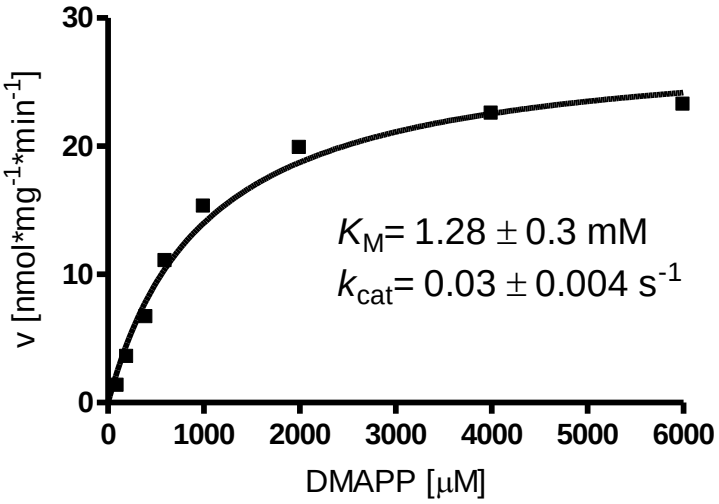


Figure S20: Determination of kinetic parameters for BAE61387 with DMAPP using 1,7-dihydroxynaphthalene (**2a**) as aromatic substrate.

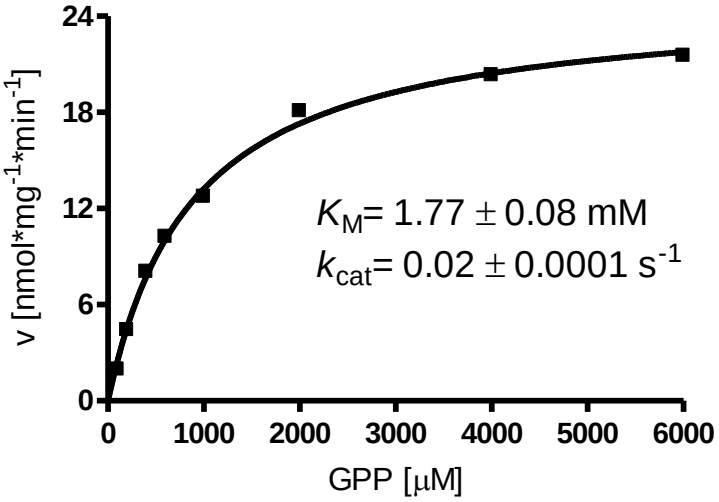


Figure S21: Determination of kinetic parameters for BAE61387 with GPP using 1,7-dihydroxynaphthalene (**2a**) as aromatic substrate.

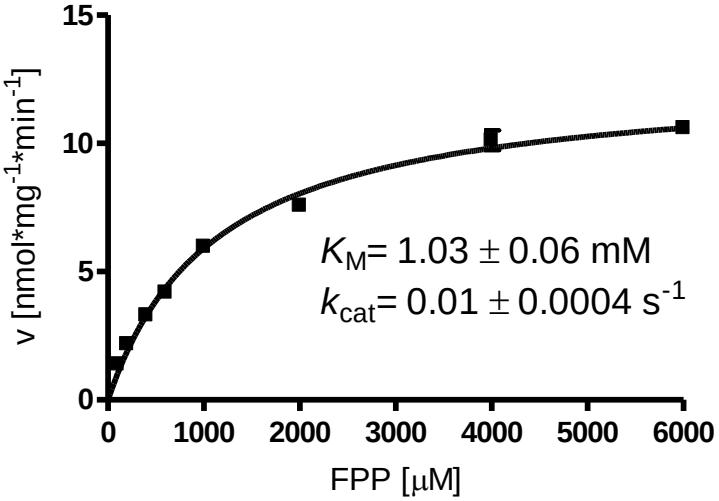


Figure S22: Determination of kinetic parameters for BAE61387 with FPP using 1,7-dihydroxynaphthalene (**2a**) as aromatic substrate.

5 Conclusions and future prospects

In the present thesis, functions of several members of the DMATS superfamily from *A. nidulans* and *A. oryzae* were elucidated.

The xanthone prenyltransferase B (XptB) from *A. nidulans* was characterised biochemically after heterologous expression and purification of the recombinant protein from *E. coli*. Our results contributed to the vital discussion about how the diprenylated xanthones are formed in *A. nidulans*. The acceptance of xanthones, but not benzophenones, by XptB as substrates, was clearly demonstrated. The prenylation reaction catalysed by XptB therefore takes place after formation of the xanthone scaffold. XptB is most likely not the prenyltransferase responsible for the prenylation of arugosin H, which was another suggested substrate. Unfortunately, for XptA no enzyme activity was observed, which would provide even deeper insights into the biosynthetic pathway of the prenylated xanthones from *A. nidulans*. However, XptB was shown to accept several xanthone derivatives as substrates, catalysing regiospecific O-prenylations, and thereby proved its value for chemoenzymatic synthesis of prenylated xanthones, a compound class with many interesting biological and pharmaceutical activities.

Furthermore, two DMATS prenyltransferase genes, ANID_10289 and ANID_11080 (*nptA*) from *A. nidulans*, were also cloned and the plasmids are available for further experiments. Suggested experiments are subcloning of the genes into the expression vectors pQE60 and pQE70, respectively. The overproduction and purification of the recombinant proteins from *E. coli* would allow biochemical investigation of both enzymes. NptA was shown before to be responsible for the prenylation of a tetracyclopeptide at an L-kynurenine moiety with DMAPP, yielding nidulanin A. It is speculated that the prenyltransferase may accept L-kynurenine-containing cyclic oligopeptides, which are suggested substrates for this enzyme.

The report about the acceptance of GPP by a member of the DMATS prenyltransferases during preparation of this thesis prompted us to prove the acceptance of prenyl donors with larger carbon chain by several members of the DMATS superfamily. From the chosen enzymes, only AnaPT from *N. fischeri* accepted as prenyl donor GPP besides the common prenyl donor DMAPP. The use of GPP instead of DMAPP resulted in a shift of the prenylation position from C-3 to C-6 or C-7 of the indole ring of six tryptophan-containing cyclic dipeptides. It was speculated, that the observed shift of prenylation position was caused by a different spacial arrangement of the prenyl donors inside the reaction chamber of AnaPT. In the case of DMAPP, the C-1' is coordinated already at such a distance to the C-3 of the indole moiety that the nucleophilic attack of this C-atom has to take place at C-3' of the DMAPP cation after cleavage

of the pyrophosphate. If now the larger geranyl moiety binds in the same orientation as DMAPP, the distance between C-1' of GPP to C-3 of the indole is even larger, and the only available position for a nucleophilic attack seems to be the benzene ring. The acceptance of both prenyl donors by a member of the DMATS was never before observed and proved the applicability of AnaPT for the production of geranylated aromatic compounds.

From these findings it was further speculated, that members from this enzyme superfamily might also accept DMAPP, GPP and FPP as prenyl donors. The putative DMATS prenyltransferase gene AO090102000322 was identified in the genome of *A. oryzae* RIB40. For this enzyme, no natural substrate was predictable by sequence analysis. The deduced protein BAE61387 was overproduced and purified from *E. coli* and incubated with a large number of potential substrates. It was found that BAE61387 accepted DMAPP, GPP and FPP in the presence of hydroxynaphthalene derivatives. The enzyme catalysed regiospecific C-prenylations with all three prenyl donors and proved itself useful for the attachment of dimethylallyl, geranyl and farnesyl moieties to hydroxynaphthalenes. This prenyl donor promiscuity was never before observed for a member of this enzyme superfamily.

From *A. oryzae*, the further two putative DMATS genes AO090701000600 and AO090020000527 were amplified by PCR and cloned into cloning vectors. These plasmids are also available for further experimentation. Suggested experiments are subcloning of both genes in expression vector pQE70. Overproduction and purification of the recombinant proteins from *E. coli* would allow biochemical investigation of both enzymes.

Further suggested experiments are testing of biological and pharmaceutical activities of all obtained prenylated aromatic compounds.

6 References

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7 Appendix

Table 7.1 Plasmids prepared during this thesis.

plasmid	description
pDP003	AO0901020000322 (accession number XM_001822468 in GenBank) in pGEM-T Easy, containing the 1351 bp PCR product amplified from genomic DNA from *A. oryzae* DSM1147 using the primer pair BAE61387_*Nco*I and BAE61387_*Bgl*II.
pDP004	Expression vector pQE60 for AO0901020000322, containing 1346 bp *Nco*I-*Bgl*II fragment of pDP003 at the same restriction sites.
pDP005	ANID_06784 (accession number XM_659296 in GenBank) in pGEM-T Easy, containing the 1361 bp PCR product amplified from genomic DNA from *A. nidulans* FGSCA4 by fusion PCR. For amplification of first exon the primers ANID_06784_*Sph*I and ANID_06784_2, for second exon ANID_06784_3 and ANID_06784_*Bam*HI were used. For fusion of the exons the primers ANID_06784_*Sph*I and ANID_06784_*Bam*HI were used.
pDP006	Expression vector pQE70 for ANID_06784, containing 1346 bp *Sph*I-*Bam*HI fragment of pDP005 at the same restriction sites.
pDP007	*xptB* (bp 445323–445526 + 445581-446753 from GenBank accession BN001302.1) in pGEM-T Easy, containing the 1382 bp PCR product amplified from genomic DNA from *A. nidulans* FGSCA4 by fusion PCR. For amplification of first exon the primers XptB_*Nco*I and XptB_2, for second exon the primers XptB_3 and XptB_*Bam*HI were used. For fusion of the exons the primers XptB_*Nco*I and XptB_*Bam*HI were used.
pDP008	Expression vector pQE60 for *xptB*, containing 1376 bp *Nco*I-*Bam*HI fragment of pDP007 at the same restriction sites.
pDP009	AO090020000527 (accession number XM_001824776 in GenBank) in pGEM-T Easy, containing the 1284 bp PCR product amplified from genomic DNA from *A. oryzae* DSM1147 by fusion PCR. For amplification of first exon the primers BAE63695_*Sph*I and BAE63695_2, for second exon BAE63695_3 and BAE63695_*Bam*HI were used. For fusion of the exons the primers BAE63695_*Sph*I and BAE63695_*Bam*HI were used.
pDP011	AO090701000600 (1167 bp part of accession number XM_001823372 (bp 214-1380) in GenBank) in pGEM-T Easy, containing the 1179 bp PCR product amplified from genomic DNA of *A. oryzae* DSM1147 using the primers BAE62291_*Sph*I and BAE62291_*Bam*HI.
pDP013	ANID_10289 (accession number XM_654857 in GenBank) in pGEM-T Easy, containing the 1350 bp PCR product amplified from genomic DNA from *A. nidulans* FGSCA4 by fusion PCR. For amplification of the first exon the primers ANID_10289_ *Nco*I and ANID_10289_2 and for second exon the primers ANID_10289_3 and ANID_10289_*Bam*HI were used. For fusion of the exons the primers ANID_10289_ *Nco*I and ANID_10289_*Bam*HI were used.
pDP017	*nptA* (ANID_11080; accession number XM_676659 in GenBank) in pGEM-T Easy, containing the 1329 bp PCR product amplified from genomic DNA from *A. nidulans* FGSCA4 by fusion PCR; For amplification of the first exon the primers ANID_11080_*Sph*I and ANID_11080_2, and for the second exon the primers ANID_11080_2 and ANID_11080_*Bam*HI were used. For fusion of the exons the primers ANID_11080_*Sph*I and ANID_11080_*Bam*HI were used.

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page 129 contains personal data and is not included in the online publication.